EXAMINATION OF NUCLEUS ACCUMBENS MECHANISMS UNDERLYING THE MOTIVATION FOR PHYSICAL ACTIVITY

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by

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled:

EXAMINATION OF NUCLEUS ACCUMBENS MECHANISMS UNDERLYING THE MOTIVATION FOR PHYSICAL ACTIVITY

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To my family:

my parents Bill and Linda, my sister Natalie, as well as my extended family.

Your support and encouragement make even the most challenging endeavors attainable.

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Knowledge is like money: to be of value it must circulate, and in circulating it can increase in quantity and, hopefully, in value.

-Louis L'Amour

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LIST OF ABBREVIATIONS

ACUC, Animal Care and Use Committee

Agrp, agouti-related peptide

AMG, amygdala

ANOVA, analysis of variance

Arc, arcuate nucleus of the hypothalamus

BMI, body mass index

BW, body weight

Bdnf, brain derived neurotrophic factor

Cadm4, cell adhesion molecule 4

Cart, cocaine- and amphetamine-regulated transcript

CDC, Centers for Disease Control

Cdk5, cyclin-dependent kinase 5

Cdk5r1, cyclin-dependent kinase 5, regulatory subunit 1 (p35)

Cdk5r2, cyclin-dependent kinase 5, regulatory subunit 2 (p39)

CHD, coronary heart disease

CPM, counts per million

DA, dopamine

Darpp32, Dopamine- and cAMP-regulated neuronal phosphoprotein of 32kDa

Dat, dopamine transporter

DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin

Drd1, dopamine receptor 1

Drd2, dopamine receptor 2

Drd5, dopamine receptor 5

DNA, deoxyribonucleic acid

DXA, dual-energy X-ray absorptiometry

Dyn, dynorphin

Enk, enkephalin

FDR, false discovery rate

G, generation

Gad, glutamic acid decarboxylase

GABA, gamma-aminobutyric acid

GO, gene ontology

HFD, high fat diet

HVR, high voluntary runner

ICV, intracerebroventricular

Insr, insulin receptor

Ip, intraperitoneal

IPA, Ingenuity Pathway Analysis Software

Km, kilometer

Lepr, leptin receptor

LFD, low fat diet

LVR, low voluntary runner

Mc4r, melanocortin-4 receptor

NAc, nucleus accumbens

Npy, neuropeptide Y

NTX, naltrexone

MET, metabolic equivalent

mPFC, medial prefrontal cortex

MSN, medium spiny neuron

NFDM, non-fat dry milk

6-OHDA, 6-hydroxydopamine

Oprd1, opioid receptor delta 1

Oprk1, opioid receptor kappa 1

Oprm1, opioid receptor mu 1

PF, pair fed

Pomc, pro-opiomelanocortin

PWL, paw withdrawal latency

PVN, paraventricular nucleus

qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction

RIPA, radioimmunoprecipitation assay buffer

RNA, ribonucleic acid

RNA-seq, RNA sequencing

ROS, roscovitine

RPKM, reads per kilobase per million mapped reads

RUN, voluntary running group

SD, standard diet/chow

SDS, sodium dodecyl sulfate

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

SED, sedentary group

STAT3, signal transducer and activator of transcription-3

T2D, type 2 diabetes

Th, tyrosine hydroxylase

VEH, vehicle

VTA, ventral tegmental area

WD, Western diet

WHO, World Health Organization

Wk, week

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ABSTRACT

Physical inactivity, a primary contributor to numerous diseases including obesity, type 2 diabetes, depression, and dementia, has reached pandemic levels worldwide. Alarmingly, the percentage of individuals engaging in physical activity is low and decreasing. Accelerometry data shows that > 90% of adults fail to meet the U.S. Physical Activity Guidelines despite the excess of knowledge suggesting exercise improves health. Therefore, beginning to understand the molecular mechanisms which influence physical activity levels is imperative for the development of therapies to reduce sedentary behavior. The work presented in this dissertation made use of three independent experimental paradigms in rats to test the hypothesis that differences in the mesolimbic dopamine system associate with/cause changes in voluntary physical activity. In the first study, rats selectively bred for high (HVR) or low (LVR) voluntary wheel running distance were used to assess inherent differences in opioidergic signaling between HVR and LVR, as well as the influence of dopamine on opioid-induced changes in voluntary wheel running. Mu-opioid receptor expression and function was increased in the nucleus accumbens (NAc) of HVR compared to LVR. Likewise, naltrexone injection decreased dopamine-related mRNA expression in mesolimbic brain regions and reduced wheel running in HVR, but not LVR. Finally, lesion of dopaminergic neurons in the NAc

prevented the decrease in running following naltrexone administration in HVR, suggesting opioidergic signaling requires downstream dopaminergic activity to influence voluntary running. In the second study, the transgenerational effect of maternal Western diet (WD) on offspring voluntary wheel running was assessed. Wheel running was increased in female WD offspring from 4-7 weeks of age, but decreased running from 16-19 weeks of age, compared to offspring from chow fed dams. These age-specific changes in wheel running are associated with the up- and down-regulation of dopamine receptor 1 in the NAc at 6 and 18 weeks of age, respectively, in WD female offspring, which in turn was negatively associated with leptin receptor mRNA in the ventral tegmental area. In the third study, age-related influences on wheel running were assessed in 8 and 14 week-old rats. In addition to a ~60% reduction in running, RNA-sequencing revealed down-regulations in networks related to cAMP-mediated signaling and synaptic plasticity in the NAc from 8 to 14 weeks-old. The down-regulations of these networks was mirrored by reductions in dendritic spine density in the NAc from 8 to 14 weeks-old. Additionally, intra-NAc injection of the Cdk5 inhibitor roscovitine, a known modulator of dendritic density and dopamine signaling, dose-dependently decreased wheel running. Despite the varying experimental models used in this dissertation, these findings collectively suggest that alterations in dopaminergic signaling in the NAc associate with, and influence, voluntary physical activity.

CHAPTER 1: Introduction

Physical inactivity is a major cause of chronic diseases

In the 5th century BC, the ancient physician Hippocrates stated: "All parts of the body, if used in moderation and exercised in labors to which each is accustomed, become thereby healthy and well developed and age slowly; but if they are unused and left idle, they become liable to disease, defective in growth and age quickly." However, by the 21st century, the value of physical activity for health has drastically declined.

Physical inactivity has reached pandemic levels in the United States and now presents a major public health problem. In 1993, McGinnis & Foege (McGinnis and Foege, 1993) first classified physical inactivity as a cause of chronic diseases and death. Strong evidence shows that physical inactivity is associated with at least 35 chronic diseases, including major non-communicable diseases such as type 2 diabetes (T2D) and coronary heart disease (CHD), and premature mortality (Booth et al., 2012). Predictions by Lee et al. (Lee et al., 2012) suggest that physical inactivity is responsible for between 6-10% of T2D, CHD, and breast and colon cancer prevalence, and this percentage is further elevated for specific diseases (i.e. 30% for ischemic heart disease) (WHO, 2010). Collectively, these striking findings recently led the World Health Organization to declare physical inactivity as the fourth leading risk factor for death worldwide, responsible for ~6% of the deaths worldwide in 2008 (Lee et al., 2012; WHO, 2010). These are dumbfounding numbers and, in my opinion, are mostly unnoticed since chronic diseases associated with physical inactivity are non-communicable. For example, I speculated that the ~6 million deaths caused by physical inactivity each year are much

less publicized than the 11,315 tragic deaths from Ebola virus disease up to January, 2016.

Remarkably, the elimination of physical inactivity may be expected to increase life expectancy of the world's population by 0.68 years, making inactivity a risk factor comparable to well established risk factors such as smoking and obesity (Lee et al., 2012). For comparison, smoking reduces life expectancy by ~10 years, while extreme class II obesity (BMI > 35) with no physical activity is associated with 7.2 fewer years of life compared to the normal weight group with > 7.5 MET-hr/wk (Moore et al., 2012). In the same study of ~650,000 individuals, brisk walking for 75 min/wk or 450 min/wk was associated with gains in life expectancy of 1.8 and 4.5 years, respectively, relative to 0-MET hr/wk (Moore et al., 2012). Additionally, the associated morbidity caused by physical inactivity, including health-related quality of life as well as direct and indirect economic costs, equates to $154 to $419 per person in the United States (Chenoweth and Leutzinger, 2006). In view of its prevalence and global reach, strategies to reduce the pandemic levels of physical inactivity must be undertaken to lessen its far-reaching economic, social, and environmental consequences.

The majority of individuals are not physically active despite believing exercise is good for health

Published in 1953, Jeremy Morris conducted the first rigorous, epidemiological studies investigating physical inactivity and chronic disease risk (Morris et al., 1953). Since this pioneering report, many studies have documented health benefits of physical activity. Despite this knowledge, a Report from the U.S. Department of Health & Human

Services states that most adults and many children lead relatively sedentary lifestyles and are not active enough to achieve the health benefits of exercise despite the well-known benefits of physical activity (US Dept. of Health and Human Services, 2008). Alarmingly, the percentage of Americans engaging in physical activity is low and decreasing. Troiano et al. (Troiano et al., 2008) reported that 42% of children ages 6-11 years obtain the recommended U.S. government guidelines of 60 min/day of physical activity, whereas only 8% of adolescents (12- 17 years old) achieve this goal, as shown in Figure 1.1. In addition, adherence among adults is <5% in meeting the U.S. guidelines of obtaining 30 min/day of physical activity. From these data, it can be concluded that 88% of the total United States population (or roughly 282 out the 319-million-person population of the United States in 2014) do not meet governmental standards for physical activity. In addition, limited available questionnaire data suggests that 31% of the world's population in 2009 did not obtain the minimum recommended levels of physical activity (Hallal et al., 2012).

In light of these statistics, many investigators feel that the identification of genetic and/or orally active agents that mimic the effects of endurance exercise is a longstanding medical goal (Narkar et al., 2008). However, the expert opinion of Hawley et al. (Hawley et al., 2014) suggests that "finding ways to motivate people to adapt and maintain a physically active lifestyle will have a greater impact than searching for potential pharmacological treatments." Together, the above opinions suggest more significant efforts should be made to understand the neuromolecular mechanisms which control the motivation to be physically active.

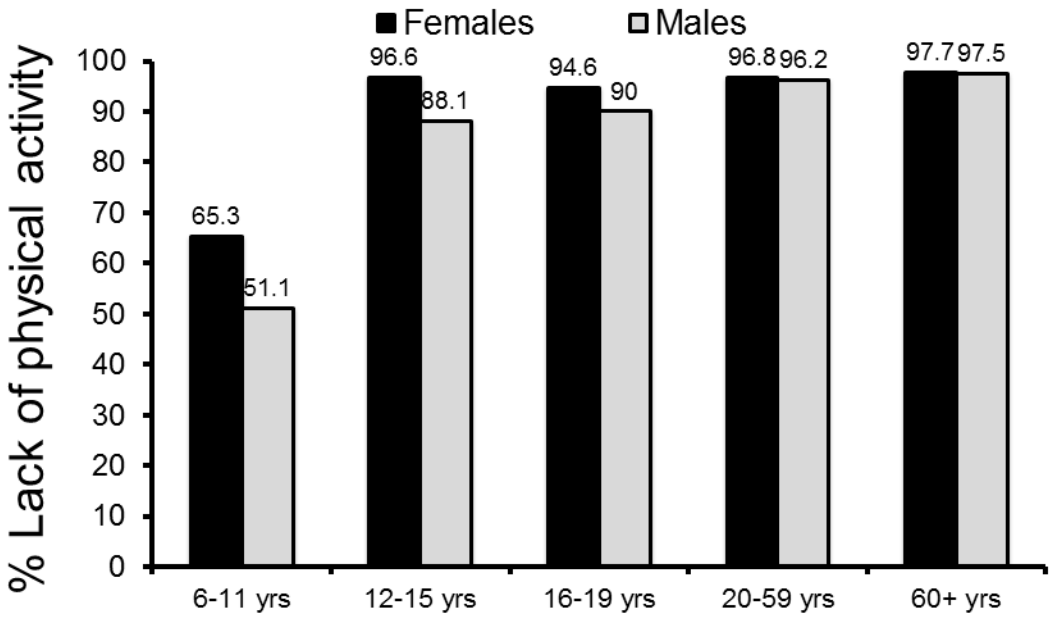

Figure 1.1. Physical activity adherence in the United States. Accelerometry data collected from 6,329 individuals showing the percentage of the U.S. population, by age, who fail to meet U.S. federal activity guidelines [adapted from Troiano et al. (Troiano et al., 2008)].

Physical activity levels decline during juvenile and adolescent life

The level of physical activity achieved at any point in the lifespan is the combination of a complex interaction among genetic, biological, psychological, and environmental factors. Several studies support the notion that physical activity levels begin to fall during adolescence. As stated above, Troiano et al. (Troiano et al., 2008) reported a ~34% decline in physical activity between 6-11 and 12-17 years of age. A 2002 accelerometer study by Trost et al. (Trost et al., 2002) observed a 40% decline in time spent performing moderate-vigorous physical activity going from the groupings 1st-3rd grades to 4th-to 6th grade, with ~9 years of age serving as the chronological age transition between groups. Additionally, the age at which the average amount of physical activity was below U.S. Physical Activity Guidelines occurred at ~15-16 years for both

sexes (Trost et al., 2002). In 2014, Wolff-Hughes et al. (Wolff-Hughes et al., 2014) analyzed data from 3,700 U.S. youth in the US 2003-2006 National Health and Nutrition Examination Survey. From the age of 6- to 11-years old, the minutes of physical activity in U.S. girls and boys dropped ~67% and ~60%, respectively (Wolff-Hughes et al., 2014). Further, at 8.7 and 10 years of age, 50% of girls and of boys, respectively, engaged in <60 min of daily intermittent moderate-vigorous physical activity. Similar findings by Rääsk et al. (Raask et al., 2015) show that pubertal boys (11.5 to 13.9 years of age) started to be less active according to accelerometer data in their pubertal period. Findings from rodent studies provide similar results.

In 1969, Jakubczak (Jakubczak, 1969) demonstrated that declines in free wheel running activity begin early in the life of rats (66 d, or ~ 9 weeks of age), and this decline in continuous through early adulthood (186 d). Additionally, studies by our lab suggest that voluntary wheel running initially declines at 8 weeks of age, a timeframe comparable to the age at which activity decreases in humans and associated with the onset of puberty (Sengupta, 2013). Importantly, there are developmental similarities between humans and rodents that allow translation of the age-specific changes of human life to both juvenile and adult rat models, as shown in Figure 1.2.

Taken together, these reports show that by ~9-11 years of age, 50% of U.S. youth are not undertaking sufficient daily physical activities for health according to U.S. Physical Activity Guidelines, and this phenomenon appears to be conserved across species. However, it is unknown whether there is a biological basis for the drop in average physical activity per day occurring between 6-11 years of age.

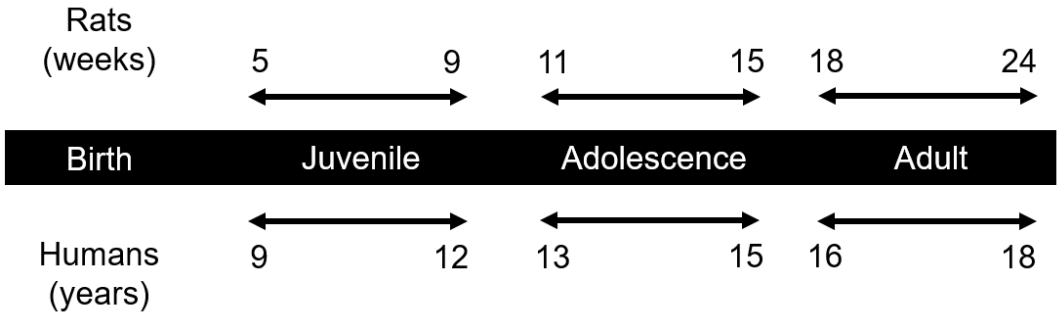

Figure 1.2. Comparison of rat and human age. The age of rats in weeks for juvenile, adolescent, and adult life compared to years from childhood to adulthood in humans (Andreollo et al., 2012; Sengupta, 2013).

Is there a biological basis for physical inactivity?

Despite the pandemic levels of physical inactivity, little is known about the underlying genetic and biological factors that may contribute to the regulation of physical inactivity behavior. While social and cultural pressures certainly influence physical activity in humans, they do not regulate these behaviors entirely. Observations as early as 1954 demonstrate that physical activity volition has a biological basis (Mayer et al., 1954). In their study, Mayer et al. (Mayer et al., 1954) noted that obese hyperglycemic mice were far less active than non-obese littermates, and that inactivity, rather than overnutrition, was the major factor. However, when obese mice are bred against mice with a so-called "waltzing gene," the increased physical activity is sufficient to prevent the development of obesity. Studies in animals and humans estimate the genetic component for physical inactivity to be between 20-80% (Festing, 1977; Kaprio et al., 1981; Lauderdale et al., 1997; Lerman et al., 2002; Lightfoot et al., 2004; Lightfoot et al., 2008). Additionally, in a recent study following 1654 twins from 420 monozygotic and 352 dizygotic same-sex twin pairs, den Hoed et al. (den Hoed et al., 2013) provided heritability estimates of physical activity levels. Results indicated that sedentary behavior

is moderately heritable in adults. Additive genetic factors (i.e., heritability) explained 31% (95% CI: 9%, 51%) of the variance in the time spent performing sedentary behavior, with the remaining variance predominantly explained by unique environmental factors.

Evolutionary perspectives also argue that while selection did not operate to cope with the detrimental effects of long-term physical inactivity, humans adapted to avoid unnecessary exertion due to limited energy supply. Lieberman (Lieberman, 2015) states that while humans evolved for regular, moderate amounts of endurance physical activity in conjunction with hunter-gather behaviors, humans are equally well evolved to be physically inactive. For example, hunter-gathers in camp spent a majority of their time sitting on the ground, doing chores, or engaging in activities that require little energy expenditure (Lieberman, 2015). The evolutionary pressures selecting to avoid excess physical activity may be best exemplified by energy balance assessments, as described by Lieberman in the following:

> "it is important to note that hunter-gatherers tend to have much smaller body masses than people in developed nations, so estimates of their active energy expenditure (TEE-RMR) relative to body mass indicate that they expend on average 30 kcal/kg/d, almost twice that of Americans, which is 17 kcal/kg/d (Lieberman, 2015). In other words, hunter-gatherers who are very physically active for only 4 to 6 h/d are still nearly twice as active as people in postindustrial economies, which explains why they are under such strong selection to be inactive as much as possible. I know of no behavioral studies on inactivity among hunter-gatherers but predict that they are just as keen to avoid physical activity."

Data on energy budgets in hunter-gathers suggests that since energy intake often matched energy expenditure (sometimes greater, sometimes less), inessential physical activity, which we often consider as exercise today, may have been maladaptive. Furthermore, humans, especially females, have an evolutionary ability to store large amounts fat to have sufficient energy stores to afford for numerous unusually energy-costly demands of human biology in pregnancy compared to other primates such as, delayed development, larger bodies and brains, and rapid reproduction rates (Lieberman, 2015). In total, strong selection pressures not only appear to shape human desires to engage in physical activity, but also physical inactivity.

Further support for a genetic component of voluntary physical activity can be found in mice bred for high voluntary wheel running (Rhodes et al., 2005), and rats bred for high or low voluntary wheel running (Roberts et al., 2013). In high voluntary running mice, single-gene quantitative trait loci (QTL) analysis has been used to identify regions of the genome that may mediate the motivation of physical activity (Lightfoot et al., 2008). In doing so, Lightfoot et al. identified specific sections of chromosome 13 associated with running distance and duration, respectively. Similarly, transcriptomic analysis of the nucleus accumbens (NAc) has identified at least 13 transcripts inherently differently expressed between high and low voluntary running rats (Roberts et al., 2014). Additional details about specific genetic contributes to voluntary physical activity/inactivity are discussed in the following sections.

While, it is necessary to investigate environmental and non-genetic factors, as well as their interactions with genetic factors, to understand fully the complex

mechanisms that contribute to the regulation of physical activity, the above examples provide strong evidence to suggest that genetic factors influence the desire to be physically active.

Nucleus accumbens, dopamine, and physical activity motivation

Although detailed mechanisms describing the neurobiology of wheel running motivation are incomplete, substantial evidence suggests the mesolimbic dopaminergic pathway, specifically the nucleus accumbens (NAc), plays an important role in determining voluntary running behavior (Knab et al., 2009; Knab et al., 2012; Knab and Lightfoot, 2010). In the mesolimbic dopamine system, dopaminergic neurons originating in the ventral tegmental area (VTA) project to various limbic nuclei including the NAc, amygdala, hippocampus, ventral pallidum, and medial prefrontal cortex (mPFC), and changes in dopamine (DA) transmission play central roles in modulating the flow of information through the limbic system (Carr et al., 1999; Kalivas and Nakamura, 1999; Napier and Maslowski-Cobuzzi, 1994; Sesack et al., 2003). These nuclei, through interconnections via dopaminergic neurons, have implications in central functions such as reward, motivation, learning, and motor movement (Smith et al., 2009). An overview of the mesolimbic DA system, as well as additional neuro-modulators of DA release and reward, is shown in Figure 1.3.

Located in ventral striatum, the NAc is a key component of the basal ganglia – a collection of subcortical nuclei that control a wide variety of psychomotor behaviors. Nearly all of the functions attributed to the NAc are intimately tied to its rich dopaminergic innervation (Nicola et al., 2000). The vast majority (90-95%) of neurons in

the NAc are GABAergic medium spiny neurons (MSNs) that predominantly receive dopaminergic input from the VTA of the midbrain (Meredith, 1999). NAc MSNs express D1-like (D1) (includes dopamine receptor (Drd) 1 and Drd5) and D2-like (D2) (includes Drd2, Drd3, and Drd4) DA receptors, with the former activating adenylate cyclase to increase cAMP and the latter inhibiting adenylate cyclase (Missale et al., 1998; Stoof and Kebabian, 1981). The collective contributions of DA receptor and downstream signaling leads to changes in gene expression, most notably the induction of immediate early genes of the Fos family (e.g. c-fos and ΔFosB) (Hiroi et al., 2002; Nestler et al., 2001; Rhodes et al., 2003) and brain derived neurotrophic factor (Bdnf) (Ernst et al., 2006).

The NAc is commonly referred to as a 'hub' for processing signals related to motivational stimuli (Salamone and Correa, 2012), making it an ideal target to identify changes in reward-related signaling that may influence behavior. Furthermore, the NAc is involved in mediating motivational behavioral processes, specifically those that include the exertion of effort and sustained task engagement (Salamone and Correa, 2012), and the NAc may act as a 'filter' or 'gate' for information traveling to and from various limbic and motor areas of the brain (Roesch et al., 2009). Since the early characterization as the neural interface between the limbic 'motivation' and motor systems (Mogenson et al., 1980), the NAc and its associated circuitry has been shown to mediate many motivating and rewarding behaviors. For example, drug self-administration (Yao et al., 2006), ethanol consumption (Barkley-Levenson et al., 2016), and food intake (Baldo et al., 2013), are influenced by the NAc.

Previous findings have established that the NAc plays an important role in determining running behavior in rodents (Knab et al., 2009; Knab et al., 2012; Knab and

Lightfoot, 2010; Waters et al., 2008). A report by Knab et al. (Knab et al., 2009) suggests that differences in D1 receptors as well as tyrosine hydroxylase (Th) mRNA, an enzyme required for catecholamine synthesis of DA, in the NAc are associated with differences in running distance between mouse strains. Similar findings by Greenwood et al. (Greenwood et al., 2011) show that voluntary wheel running is rewarding, and over time, able to alter behavior and affect the neuroplasticity of the mesolimbic reward pathway. In the same study, 6 weeks of voluntary wheel running increased Th mRNA in mesolimbic brain regions, decreased Drd2 mRNA in the NAc, and increased ΔFosB/FosB immunoreactivity, a transcription factor associated with rewarding behavior and chronic drug reward, in the NAc. Additionally, the same report by Greenwood et al. (Greenwood et al., 2011) used a conditioned place preference paradigm to show that rats spent more time in a context paired with wheel-running compared to a non-paired context, a finding that is consistent with the place preference developed in the context of either cocaine or morphine.

DA receptor expression levels in the striatum have been posited to influence physical activity, and is elevated following five continuous days of exercise (Foley et al., 2006). Mice bred by Garland for high voluntary running distance display dysfunctional dopaminergic profiles in the NAc compared to control mice (Rhodes and Garland, 2003; Rhodes et al., 2001). Further, agonism (Knab et al., 2012) and antagonism (Rhodes and Garland, 2003) of Drd1 in the NAc paradoxically both decrease wheel running in high running mice to a greater extent than in control mice. This suggests the following: 1) DA signaling in high running is optimally primed to achieve reward associated with running, 2) DA is required for wheel running motivation, and 3) animals run to achieve the

rewarding effects of DA and do not want to run when DA signaling is artificially activated. In addition, the depletion of NAc DA by 6-hydroxydopamine (6-OHDA) decreases wheel running (Robbins and Koob, 1980). Recent findings by Beeler et al. using Drd2 knockdown mice also suggest a direct link between reduced dopamine function and reduced physical activity (Beeler et al., 2016). Importantly, in this study Beeler et al. also suggest that the primary contribution of decreased Drd2 signaling to obesity is via reductions in energy expenditure rather than the initiation of compulsive overeating. Additionally, several reports have demonstrated other mesolimbic structures, such as the VTA and PFC, also contribute to the reward derived from physical activity, potentially through their interactions with the NAc (Greenwood et al., 2011; Isobe and Nishino, 2001; Rhodes et al., 2005).

Together, these data suggest the NAc is an intriguing and potentially rich loci to study biological factors that influence the motivation to be physically active. Given the infancy of this area of research and the vast ramifications of a physical inactive lifestyle, significant efforts should be made to determine potential mechanisms by which molecular changes in the NAc drive the motivation for exercise.

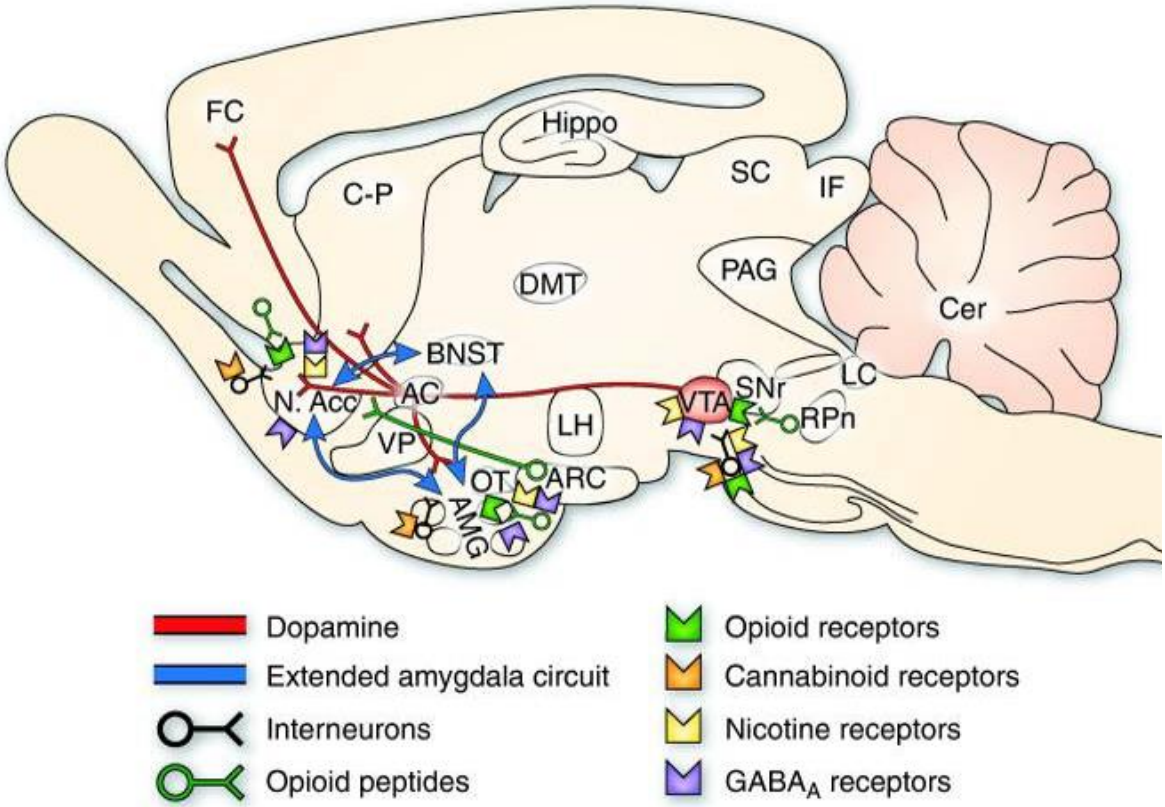

Figure 1.3. Neurocircuits implicated in reward and motivation. Sagittal section of a representative rodent brain illustrating the pathways and receptors implicated in rewarding behavior. Note the high convergence of dopaminergic inputs on the nucleus accumbens. In response to a rewarding stimulus, DA is released from the ventral tegmental area to the nucleus accumbens through direct actions on DA terminals. In addition, opioids activate opioid receptors in the ventral tegmental area, nucleus accumbens, and amygdala through direct or indirect actions via interneurons. Opioids facilitate the release of DA in the nucleus accumbens by an action either in the ventral tegmental area or the nucleus accumbens, and are also hypothesized to act independent of the DA system. Activation of γ-aminobutyric acid-A ($GABA_A$) receptors or GABA release in the ventral tegmental area or nucleus accumbens also influence both opioid peptide and DA release in the nucleus accumbens. Additionally, nicotine and cannabinoids facilitate the release of DA in the nucleus accumbens through mechanisms either in the ventral tegmental area or the nucleus accumbens. Abbreviations: AC, anterior commissure; AMG, amygdala; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; Cer, cerebellum; C-P, caudate-putamen; DMT, dorsomedial thalamus; FC, frontal cortex; Hippo, hippocampus; IF, inferior colliculus; LC, locus coeruleus; LH, lateral hypothalamus; N Acc., nucleus accumbens; OT, olfactory tract; PAG, periaqueductal gray; RPn, reticular pontine nucleus; SC, superior colliculus; SNr, substantia nigra pars reticulata; VP, ventral pallidum; VTA, ventral tegmental area (Koob and Volkow, 2010).

Opioids influence nucleus accumbens dopamine signaling

In addition to D1- and D2-like DA receptors, NAc MSNs are dense in mu- (μ), delta (δ), and kappa- (κ) opioid receptors, and opioidergic neurons in the striatum that receive DA projections for the VTA are involved in the interface between the limbic and motor systems (Depue and Collins, 1999). Like D1- and D2-like receptors, the effects of the μ-, δ-, and κ- receptor activation on downstream DA content/signaling vary. Findings by Spanagel et al. (Spanagel et al., 1990) in anesthetized rats suggest that selective agonism of the μ- and δ- opioid receptor in the NAc increase DA release while, in contrast, κ- receptor opioid agonism in the NAc decreases DA release. The opposing actions from the bi-functional opioid receptors are further influenced by two separate pathways in striatal GABAergic MSNs, as follows: 1) the striatonigral "direct" pathway express dynoprphin and D1-like dopamine receptors (Gerfen and Young, 1988); and 2) on the other hand, neurons in the striatopallidal "indirect" pathway express enkephalin and D2-like dopamine receptors (Schiffmann et al., 1991). These two pathways are thought to serve opposing roles for processing reward with direct pathway activation promoting reward and indirect pathway activation reducing reward (Kravitz et al., 2012).

Opioids are known to influence many rewarding behaviors, with several notable examples being drugs of abuse, ethanol consumption, and feeding behavior. Daily injection of the μ-opioid receptor agonist morphine increases food consumption in rats (Baldo et al., 2010). In contrast, Levine et al. (Levine et al., 1985) demonstrated that the opioid receptor antagonist naloxone (which has the greatest affinity for the μ-receptor) decreases food intake in rats. Similar findings have been reported with the rewarding behavior of ethanol consumption (Valenta et al., 2013). Interestingly, modulation of the

μ-opioid receptor appears to influence ethanol intake in a DA-dependent mechanism in the NAc, as determined by a measurement of extracellular DA *in vivo* using microdialysis probes inserted into the NAc (Valenta et al., 2013), but influence feeding behavior independent of DA (Will et al., 2006). Likewise, these distinctions in opioidergic signaling activity in the NAc have been posited to modulate the degree of reward associated with physical activity (Gerfen and Young, 1988), as described next.

Opioids influence physical activity motivation

Minimal attention has focused on the relationship between opioids and voluntary wheel running. However, the limited literature suggests opioidergic signaling in the ventral striatum and NAc influences voluntary wheel running. Specifically, mice selected for high wheel running have decreased κ-opioid receptor (Oprk1) mRNA expression in the dorsal striatum compared to non-selected mice (Mathes et al., 2010). In the study described in a previous section by Greenwood et al. (Greenwood et al., 2011), 6 weeks of wheel running up-regulated δ-opioid receptor (Oprd1) mRNA expression in the NAc. Furthermore, intraperitoneal injection of the opioid receptor antagonist naloxone has been shown to suppress wheel running in rats (Sisti and Lewis, 2001). Other published wheel running studies in rodents and hamsters similarly demonstrate that both opioid antagonists and agonists initially suppress running behavior (Schnur, 1985; Schnur and Barela, 1984; Schnur et al., 1983; Sisti and Lewis, 2001). Intriguingly, in one of these reports, repeated administration or recovery from the opioid agonist morphine later increased running to hyperactive levels (Schnur, 1985; Schnur et al., 1983; Sisti and Lewis, 2001). However, the authors of this study noted one limitation in that the same

cohort of rats was used for three separate experiments and the authors could not rule out the possibility that either drug sensitization or tolerance could account for their paradoxical data. Nonetheless, this evidence suggests that endogenous and exogenous opioid action in the NAc and striatum influence wheel running behavior.

Several reports have demonstrated that the opioid receptor ligands enkephalin (Enk) and dynorphin (Dyn) also influence wheel running behavior. In rats selectively bred for high (HCR) and low running capacity (LCR), which also display high and low wheel running behavior, respectively, HCR rats expressed less Enk mRNA than LCR rats in the NAc (Monroe et al., 2014). Additionally, these expression patterns were not altered after three weeks of wheel running, leading the authors of this study to speculate that differences in NAc Enk may influence inherent differences in wheel running (Monroe et al., 2014). Similarly, Enk projections from the ventral striatum have been shown to inhibit motivation-based locomotion (Haber, 2011), and low Enk mRNA is indicative of increased dopaminergic tone (Nikoshkov et al., 2008). Both Dyn and Enk have crucial roles in regulating the transcription factor ΔFosB. Werme et al. (Werme et al., 2002) found that running wheel access for 30 days reduces ΔFosB in NAc Enk-containing neurons, and selective overexpression of ΔFosB in striatal Enk-containing neurons decreased running compared to non-transgenic mice. For the opposing Dyn-pathway, in the study above by Werme et al., it was observed that 30 days of wheel access increased ΔFosB in NAc Dyn-containing neurons. Further, ΔFosB overexpression in striatal Dyn-containing neurons increased running, suggesting that dynorphin may regulate the incentive motivation or reward associated with wheel running (Werme et al., 2002). Additionally, multiple findings indicate lesioning of dopaminergic neurons decreases

Dyn expression in striatal neurons and this effect is mediated by D1-like receptors (Engber et al., 1992; Gerfen et al., 1990; Gerfen et al., 1991).

The neurochemical mechanisms by which opioids influence wheel running may be shared with the mechanisms by which opioids affect drug addiction. Access to voluntary running wheels for either 11 or 30 days resulted in the self-administration of significantly fewer doses of the Oprm1 agonist morphine in an operant lever-pressing task compared to sedentary control rats (Hosseini et al., 2009). Further, the development of conditioned place preference to the effects of morphine were precluded after as few as 8 days of wheel running (Lett et al., 2002) Given that the naturally rewarding properties of morphine are attenuated by voluntary physical activity, it is likely that the reward/value derived from physical activity either outcompetes, or substitutes for, the reward of drugs. These findings make studies designed to test the beneficial influences of opioids on wheel running behavior in reference to drug addiction very intriguing given the plethora of data concerning opioids and drug addiction.

Nucleus accumbens dopaminergic signaling diminishes during adolescence and adulthood

As described above, physical activity levels first decline near the onset of puberty in both humans and rodents. While complex factors such as cellular energy production, reductions in circulation and removal of waste products, alterations in cellular membrane trafficking, and alterations in muscle structure and innervation would impact the ability to locomote, this dissertation will more thoroughly consider possible neurobiological

mechanisms that impact not only the ability to be physically active, but additionally the motivation to be physically active.

Findings by Andersen (Andersen, 2002) in the NAc and striatum of rats demonstrate that at puberty (7 wks of age), cAMP production in the NAc is ~35% higher relative to adulthood (14 wks of age) and forskolin-stimulated cAMP is ~300% higher at 7 wks compared to 14 wks of age. Moreover, changes in forskolin-stimulated cAMP accumulation in the NAc were parallel to the decline in locomotor activity between 7 and 14 wks of age. The significance of cAMP is that it demonstrates bidirectional properties in NAc MSNs, with D1-like and D2-like receptors increasing and decreasing cAMP, respectively. The same report also reported an age-dependent decrease in Drd1 mRNA in the NAc in striatum from 7 to 14 wks of age. This finding led the authors to conclude that age-dependent pruning of D1-like receptors in the NAc and striatum may mediate changes in susceptibility to various behavior beginning at the time of puberty.

Similar findings have been reported for the Drd2. Age-related decreases in Drd2 concentration in the striatum represent one of the greatest neurochemical changes in aged brains, according to Roth (Roth, 1995), and the loss of this receptor with increasing age has been noted in rodents, monkeys, and humans (Roth and Joseph, 1994). However, these findings were in middle-aged to older adult animals. Additionally, the number of dopaminergic neurons in the NAc and striatum decreases with increasing age, with the human decline estimated at a rate of 5-10% per decade (Naoi and Maruyama, 1999). Conversely, similar declines in total DA content were not observed across a similar timeframe (Joseph and Roth, 1992).

Nonetheless, the loss of DA receptors and DA neurons with advancing age have been associated with decreased motivation for various behaviors, and have been hypothesized to underlie age-related declines in the motivation to be physically active (Ingram, 2000). Therefore, any search for the biological basis of the age-related decline in physical activity motivation should place significant emphasis on examining circuity related to the DA reward system.

Maternal nutritional status influences offspring nucleus accumbens dopamine signaling as well as spontaneous activity

In 1956, Waddington provided the first conclusive evidence demonstrating that the inheritance of a characteristic can be altered in response to an environmental stimulus, a finding often associated with the origin of epigenetics (Waddington, 1956). Since Waddington's initial observation, multiple environmental stimuli have been shown to give rise to progeny with altered genetic and phenotypic profiles.

Calorie dense, high-fat diet (HFD) or Western diet (WD) is well-recognized for increasing the risk for obesity, as well as the well-described negative health consequences of obesity such as increased risk for T2D, CVD, hypertension, and dyslipidemia, among others. In addition, maternal HFD consumption during pregnancy and nursing appears to promote genetic changes in the offspring. For example, exposure to HFD during prenatal development increases the susceptibly of the offspring to developing obesity and metabolic disease, in turn leading to further transgenerational disease transmission (Boerschmann et al., 2010; Mingrone et al., 2008). Alarmingly, in the United States the incidence of obesity among pregnant women ranges from 20-38% (Yogev and Catalano,

2009). Diabetes during pregnancy also increases the risk for obesity in both children (Mehta et al., 2012) and in adults (Lawlor et al., 2011).

In addition to disease risk, maternal HFD is postulated to influence offspring physical activity levels. Rowland (Rowland, 1998) proposed that through components related to energy balance control, an "activity-stat" may regulate the propensity for physical activity. This "activity-stat" is speculated to be influenced by environmental factors during critical pre- and post-natal periods of development. For example, a study by Li et al. (Li et al., 2013) using the small-litter mouse model of early postnatal overnutrition, adult offspring from small-litters have reduced spontaneous physical activity and energy expenditure. In addition, hyperleptinemia during pregnancy in mice has been shown to increase locomotor activity in adult offspring, with female mice displaying greater responses to hyperleptinemia compared to male mice (Pollock et al., 2015). Paradoxically, studies in rats show that both maternal food restriction and HFD decrease cage activity levels in adulthood (Cunha Fda et al., 2015; Vickers et al., 2003). In addition to nutrition, energy expenditure during pregnancy may also influence offspring physical activity levels. Recently, Eclarinal et al. (Eclarinal et al., 2016) demonstrated in mice that access to voluntary running wheels during pregnancy increases voluntary wheel running in the offspring up to 300 days of age. Surprisingly, while these findings collectively suggest that over/undernutrition during pregnancy affects physical activity levels in the offspring, the mechanisms remain largely unstudied.

One potential mechanism by which HFD during pregnancy in rodents may influence physical activity levels in offspring is via HFD-induced alterations in the mesolimbic DA system. Data from the Reyes lab shows that following HFD during

pregnancy and lactation, Drd1 and Drd2 mRNA expression in the NAc are reduced in adult offspring, while mRNA expression of dopamine transporter (Dat) and Oprm1 are increased (Vucetic et al., 2010). The differences in mRNA expression appear to be the result of altered epigenetic patterns leading to transgenerational dysfunction in the DA and opioid-related systems (Vucetic et al., 2012). Importantly, HFD effects on offspring DA and opioids appear age-dependent. Following maternal 'junk-food' (e.g. chocolate, peanut butter, biscuits, etc.) exposure during pregnancy and lactation, Ong & Muhlhausler (Ong and Muhlhausler, 2011) noted that Oprm1 mRNA was increased at 6 weeks of age but decreased at 3 months of age compared to standard chow-fed offspring, while the inverse was observed for Dat mRNA. Along these lines, a number of laboratories have identified behavioral changes indicative of dopamine dysregulation following maternal HFD or junk-food. For example, offspring of HFD fed dams have increased preference for fatty and sugary foods (Ong and Muhlhausler, 2011), and increased operant responding (i.e. working harder) for high-fat pellets (Naef et al., 2011).

Along these lines, leptin suppresses the rewarding effects of wheel running in mice via activation of signal transducer and activator of transcription-3 (STAT3) signaling in mesolimbic DA neurons, an effect which likely increases DA overflow and function in the NAc, suggesting that leptin may influence the motivational and rewarding effects of wheel running (Fernandes et al., 2015). Observations that serum leptin levels inversely correlate with marathon run-time, after adjusting for BMI (Bobbert et al., 2012) and running performance (time and speed) in mice bred for high voluntary running (Girard et al., 2007) further suggest that leptin may impact the motivational and rewarding effects of running. In addition, numerous studies have identified leptin as a key

determinant of physical activity levels. In leptin-deficient patients during the fed-state, acute leptin increases locomotor activity (Farooqi et al., 1999; Licinio et al., 2004). In leptin deficient *ob/ob* mice, acute subcutaneous leptin replacement increases wheel running during the fed state, while no effect is observed in wild-type mice (Morton et al., 2011). Although the previous examples describing the influences of leptin on physical activity are unrelated to maternal-offspring transmission, it suggests alterations in metabolism (such as HFD/WD consumption) could drive changes in VTA and NAc DA signaling, which in turn could change physical activity levels.

Similarly, changes in the hypothalamic systems involved in the homeostatic regulation of feeding and spontaneous activity following maternal HFD during pregnancy and lactation are documented. Maternal HFD increases mRNA expression of the orexigenic peptides galanin, enkephalin, and dynoprphin in the paraventricular nucleus (PVN), as well as neurogenesis of orexigenic neurons, which may increase obesity risk through overeating or reduce physical activity (Chang et al., 2008). In addition, abnormalities in proopiomelanocortin (Pomc), neuropeptide Y (Npy), and melanocortin-4 receptor (Mc4r) expression in the arcuate nucleus of the hypothalamus (Arc) have been reported in pups from obese dams (Chen and Morris, 2009; Grayson et al., 2010). Collectively, the above findings suggest that changes in reward-associated and/or homeostatic neurocircuitry could influence changes in physical activity levels following maternal HFD.

A novel model of physical activity motivation: selective breeding for high or low voluntary running behavior

A review by Bauman et al. (Bauman et al., 2012) identified 75 biological and demographic factors correlated with the motivation to be physically active. However, data from these cross-sectional and longitudinal studies have considerable variation in estimates of genetic heritability and do not present any evidence supporting causal roles by which candidate genes control physical activity levels. Further, complex behaviors, such as physical activity motivation, are likely mediated by a network of genes rather than single genes or allelic variations. This complexity makes selective breeding for a desired trait a more biologically-valid method to study the traits complex phenotype, rather than studying the effects of single gene knock-out or knock-in models (Rhodes et al., 2005).

In light of the roughly 97% of U.S. adults and 92% of adolescents not meeting US daily physical activity guidelines for 30 and 60 min, respectively (Troiano et al., 2008), our lab developed selectively bred lines of rats for low vs. high voluntary running distance, LVR and HVR, respectively. This selective breeding model was developed similar to that of Swallow et al. (Swallow et al., 1998) for HVR mice and that of Koch and Britton (Koch and Britton, 2001) for low and high aerobic capacity rats. In brief, this selection resulted in a founding population of 159 outbred Wistar rats (80 male and 79 female) subsequently selected for high and low voluntary running distance in wheels. This selection resulted in a 13 family HVR and a 13 family LVR line (Roberts et al., 2013). By Generation (G) 9, HVR rats ran ~10-fold greater distances, at a higher speed, than did LVR rats, as shown in Figure 1.4 (Roberts et al., 2013). Interestingly, minimal inherent differences in peripheral factors such as the skeletal musculature quality and body fat proportion exist between the HVR and LVR lines, suggesting differences in

brain circuitry may account for some of the drastic differences in running distance (Roberts et al., 2013).

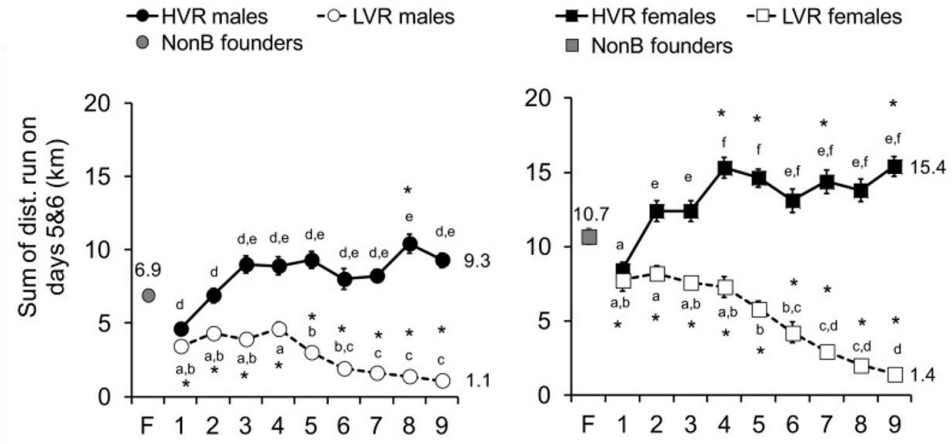

Figure 1.4. Divergence of wheel running in HVR and LVR lines. Data are from days 5 and 6 (selection criteria) for male (left) and female (right) HVR (black) and LVR (white) rats from generations 1-9. Non-breeding founder Wistar rats (F; gray) were used as a template for comparison. Symbols: $^{a\text{-}f}$ denotes a significant difference from generation to generation ($p < 0.05$). * denotes statistical difference from non-breeding founders ($p < 0.05$). † denotes statistical difference between HVR and LVR within a given generation ($p < 0.05$). Adapted from Roberts et al. (Roberts et al., 2013).

Our labs' previous findings using the HVR and LVR lines suggest intrinsic differences in NAc DA signaling pathways influence the ~10-fold differences in running behavior. By G 4-5, agonism and antagonism of Drd1 in the NAc decreased wheel running in HVR, but not LVR, rats, suggesting rats predisposed to run high nightly distances may quickly develop a rewarding response to exercise due to an optimal D1-like receptor signaling pathway in the NAc (Roberts et al., 2012). Subsequent RNA-seq studies in G8 by Roberts et al. (Roberts et al., 2014) demonstrated that 6-days of wheel running produced marked increases in transcripts in the NAc related to DA signaling in HVR rats, while transcripts in the DA pathway of LVR rats were non-responsive to wheel running. HVR rats also inherently possess more mature MSNs than LVR which possibly contributes to high and low running distances in the HVR and LVR lines,

receptively (Roberts et al., 2014). Further, in the same study by Roberts et al. (Roberts et al., 2014), 13 transcripts in the NAc identified by RNA-sequencing and Ingenuity Pathway Analysis could represent inherent genetic differences between HVR and LVR lines, providing evidence for the inheritance of genes related to physical inactivity. Similarly, differences in opioids have been reported between the HVR and LVR lines.

In a recent study, I determined that Oprm1 mRNA in the NAc was intrinsically 3-fold greater in HVR compared to LVR rats (Ruegsegger et al., 2015). In the same study, agonism and antagonism of these receptors decreased running distance in HVR, but not LVR rats. While paradoxical, the results suggest HVR express high running levels mediated by an increase in motivation driven, in part, by elevated NAc opioidergic signaling. Additional findings from this study suggested the influences of Oprm1 modulation on running distance could be dependent on NAc DA (Ruegsegger et al., 2015). Likewise, my previous findings show that prodynorphin (Pdyn) and Drd1 mRNA expression are intrinsically increased in HVR vs. LVR rats. Together, my results imply the striatonigral "direct" pathway influences wheel running behavior, and the contributions of opioidergic signaling on wheel running could be mediated through DA. Like the findings described in previous sections, these data strongly suggest the importance of NAc DA and opioids for the motivation to be physically active.

Aims of dissertation research

Understanding the neuromolecular mechanisms contributing to the motivation to be physically active is highly important. Unraveling these mechanisms may have profound implications on our future lifestyle, and may potentially lead to strategies to

reduce sedentary behavior and its associated chronic disease that is acknowledged in the U.S. Federal Physical Activity Guidelines Advisory Committee Report, 2008 (US Dept. of Health and Human Services, 2008). Thus, the aims and general hypothesis of this dissertation were the following:

Overall objective: to investigate the relationship between the mesolimbic reward pathway, specifically the NAc, and the motivation for physical activity, as assessed by voluntary wheel running, an accepted surrogate for motivation to be physically active.

Overall hypothesis: reduced dopamine signaling in the NAc associates with/contributes to lower motivation for physical activity.

This research objective and hypothesis was tested using three independent strategies, as described next:

Study 1: assess the necessity of the dopaminergic system on μ-opioid receptor driven changes in voluntary running in HVR and LVR rats.

Previously, our lab has shown that Oprm1 mRNA is elevated in HVR vs. LVR, and that modulation of Oprm1 in the NAc decreases wheel running in HVR without an effect in LVR. These prior data also suggest that the influence of Oprm1 on running behavior may be mediated potentially through DA action in the NAc. Therefore, the following original experiments were performed:

1) Extend previous findings on intrinsic Oprm1 mRNA differences between HVR vs. LVR to the protein and functional (electrophysiological) level
2) Determine if intraperitoneal (ip) injection of the opioid receptor antagonist naltrexone (20mg/kg) decreases the expression of mRNAs indicative of DA action in the NAc and VTA in HVR and/or LVR rats.
3) Determine if ip injection naltrexone (10, 20mg/kg) decreases wheel running in HVR and/or LVR rats.
4) Determine if DA neuron lesion with 6-OHDA into the NAc reduced wheel running to similar extent as naltrexone in HVR rats.

Study 2: determine the extent to which maternal Western diet (WD) (42% kcal from fat, 27% kcal from sucrose) influences transgenerational offspring physical activity, and NAc, VTA, and Arc mRNA expression in juvenile and adult offspring.

A previous report has identified changes in offspring physical activity in adulthood of HFD- and WD-fed dams (Cunha Fda et al., 2015); however, to my knowledge, no studies have attempted to identify molecular factors that associate with, and that may regulate, voluntary activity in these offspring. These effects have also never been assessed beyond the initial offspring generation. The following experiment was performed:

1) Assessment of voluntary wheel-running and home-cage locomotor activity, NAc, VTA, and Arc mRNA expression, and NAc Drd1 and Drd2 protein levels in 6 wk-old (juvenile) and 18 wk-old (young adult) was performed in

offspring from ND or WD fed dams. Additionally, F_1 generation rats within the ND or WD lines were bred, without treatment, and voluntary wheel running along with NAc and VTA mRNA expression were measured in F_2 generation offspring.

Study 3*: Aim 1:* examine the NAc of wheel running and sedentary rats at 8 and 14 weeks of age to identify transcripts indicative of the inherent age-dependent decreases in voluntary physical activity. *Aim 2:* to determine the extent to which Cdk5 inhibition alters wheel-running behavior.

The molecular factors that associate with or cause the onset of the lifetime decline in physical activity in adolescence are unknown; however, it is hypothesized NAc DA plays at least some role. The health ramifications of preventing or slowing age-related declines in physical activity are enormous. Therefore, I completed the following:

1) Perform transcriptomic analysis of the NAc of wheel running at 8 and 14 wk rats, ages at which wheel running was at its maximum and had significantly declined from maximum, respectively. Bioinformatics was used to generate hypotheses for follow-up studies to test the relationships between specific molecules/structural measurements with the age-dependent decline in running.
2) Determine if NAc infusion of the Cdk5 inhibitor roscovitine decreases running distance.

CHAPTER 2: Mu-opioid receptor inhibition decreases voluntary wheel running in a dopamine-dependent manner in rats bred for high voluntary running

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ABSTRACT

The mesolimbic dopamine and opioid systems are postulated to influence the central control of physical activity motivation. We utilized selectively bred rats for high (HVR) or low (LVR) voluntary running behavior to examine 1) inherent differences in mu-opioid receptor (Oprm1) expression and function in the nucleus accumbens (NAc), 2) if dopamine-related mRNAs, wheel-running, and food intake are differently influenced by intraperitoneal (i.p.) naltrexone injection in HVR and LVR rats, and 3) if dopamine is required for naltrexone-induced changes in running and feeding behavior in HVR rats. Oprm1 mRNA and protein expression were greater in the NAc of HVR rats, and application of the Oprm1 agonist [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) to dissociated NAc neurons produced greater depolarizing responses in neurons from HVR versus LVR rats. Naltrexone injection dose-dependently decreased wheel running and food intake in HVR, but not LVR, rats. Naltrexone (20mg/kg) decreased tyrosine hydroxylase mRNA in the ventral tegmental area and Fos and Drd5 mRNA in NAc shell of HVR, but not LVR, rats. Additionally, lesion of dopaminergic neurons in the NAc with 6-hydroxydopamine (6-OHDA) ablated the decrease in running, but not food intake, in HVR rats following i.p. naltrexone administration. Collectively, these data suggest the higher levels of running observed in HVR rats, compared to LVR rats, are mediated, in part, by increased mesolimbic opioidergic signaling that requires downstream dopaminergic activity to influence voluntary running, but not food intake.

INTRODUCTION

Identification of neuromolecular mechanisms influencing voluntary, physical activity behaviors is of paramount importance. Accelerometry measurements suggest ~90% of American adults do not meet US physical activity guidelines (Troiano et al., 2008). These statistics are concerning given that physical inactivity significantly increases risk for premature mortality, chronic disease, cognitive dysfunction, and diminished quality of life (Booth et al., 2012). While environmental factors influence physical activity, human and rodent studies show a robust genetic component for physical activity levels (Bauman et al., 2012; den Hoed et al., 2013; Lightfoot et al., 2004; Swallow et al., 1998). Therefore, elucidation of inherited genes that dictate physical activity levels will be key to developing more effective therapies to combat chronic ailments associated with sedentary lifestyles. To address initial needs for gene identities, we developed a polygenic selectively bred rat model to study the complex biology of high and low motivation for voluntary running. Here, we tested the hypothesis that inherited dopaminergic and mu-opioid systems mediate intrinsic voluntary running behavior in rats selectively-bred for high (HVR) or low (LVR) voluntary wheel-running (Roberts et al., 2014).

Mesolimbic system components, specifically dopaminergic signaling in the nucleus accumbens (NAc), are important modulators of voluntary running behavior (Knab and Lightfoot, 2010). The NAc acts as a ‘filter’ and/or ‘amplifier’ of information passing between various limbic, cortical, and motor areas, suggesting the NAc is instrumental in orchestrating behavioral processes related to motivation (Salamone and Correa, 2012). Indeed, we and others show that dopaminergic system modulation in the

NAc decreases wheel running (Rhodes and Garland, 2003; Roberts et al., 2012). Likewise, STAT3 knock-out in dopamine neurons in the ventral tegmental area (VTA) leads to reductions in dopamine overflow and function in the NAc and increased wheel running (Fernandes et al., 2015).

As the mesolimbic dopamine system is regulated, in part, by opioidergic signaling (Pierce and Kumaresan, 2006), we hypothesized that divergence in voluntary running phenotypes between HVR and LVR rats is due to inherited differences within the opioidergic system. This hypothesis was based on other studies which demonstrated that increased striatal enkephalin mRNA is associated with reduced wheel running (Monroe et al., 2014), and that injection of the opioid receptor antagonist naloxone suppresses wheel running (Sisti and Lewis, 2001). Further, we have observed that our HVR rats are more responsive to opioid receptor modulation in the NAc, and that mu-opioid receptor (Oprm1) is up-regulated in the NAc of HVR compared to LVR rats (Ruegsegger et al., 2015).

Given that interactions between the opioid and dopamine systems may influence voluntary running, we further examined HVR and LVR rats to 1) corroborate and extend upon differences in Oprm1 expression and function, 2) assess the influence of systemic naltrexone injection on mRNA expression of transcripts essential for dopamine action, 3) determine the extent to which systemic naltrexone injection influences wheel running and free-feeding behavior, and 4) determine the extent to which 6-hydroxydopamine (6-OHDA) lesion of dopaminergic neurons in the NAc influences natural, and naltrexone-induced changes in, running distance of HVR rats.

MATERIALS AND METHODS

Experimental Animals

Development and proliferation of the HVR and LVR lines are previously described (Roberts et al., 2013). Female rats from the 12^{th}-17^{th} generations of HVR and LVR lines were used in this study. We used female rats due to the fact that females usually run further than males, and may thus have greater responses to experimental treatments (Jones et al., 1990; Pitts, 1984). Additionally, another advantage to using female rats is that their body mass plateaus, minimizing the effect of continued body mass growth in male rats; our recent publications have employed female rats; and using female rats balances the predominance of male rodents in the literature. Chow (Formulab 5008, Purina) and water were provided *ad libitum*. Running distance was recorded using Sigma Sport BC 800 bicycle computers (Cherry Creek Cyclery, Foster Falls, VA). Given the potential influences of wheel running on our outcome measures, no screening strategies were used to verify phenotype in experiments conducted using sedentary rats. The Institutional Animal Care and Use Committee at the University of Missouri approved all procedures. Animals were housed in AAALAC-accredited research animal facilities. The authors used laboratory animals following the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1996). All efforts were made to minimize animals suffering and to reduce the number of animals used.

Experiment 1

Inherent differences in Oprm1 expression and function, and pain responses (primarily an opioid-derived outcome) were assessed in sedentary (group-housed without running wheels) HVR and LVR rats. At 8 wks of age, rats were sacrificed at 1700 h with carbon dioxide asphyxiation. This time-point was two hours prior to the onset of the dark cycle, and chosen to match previous findings (Ruegsegger et al., 2015). Estrous cycle was not controlled for in this experiment. Oprm1 mRNA (n = 6-7) was measured in the NAc (core and shell), VTA, medial prefrontal cortex (PFC), caudate putamen (here forth referred to as the dorsal striatum (DS)), arcuate nucleus of the hypothalamus (Arc), and hippocampus (Hippo). These regions were assessed due to their strong innervation by opioidergic and dopaminergic neurons (Mansour et al., 1995). Oprm1 protein levels (n = 6-8) were measured in NAc (core and shell). Additionally, Oprm1 protein was assessed in HVR and LVR rats (n = 4) in regions with high [lateral and medial habenula (Hb)] and low [external cuneate nucleus (ECN)] Oprm1 expression (Erbs et al., 2015). These regions were selected to provide some additional validity to the selectivity of the Oprm1 antibody. Electrophysiological (n = 6-11) responses to the mu-opioid receptor agonist [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) (Sigma) were ascertained in NAc neurons isolated from HVR and LVR rats. Additionally, paw-withdrawal latency (PWL) to thermal stimulation was examined in 6-wk-old sedentary HVR (n = 11) and LVR (n = 12) rats.

Experiment 2

The effects of opioid antagonism were determined on neuronal activation and dopaminergic gene expression in similar brain regions to those assayed in Experiment 1. Eight-week-old sedentary HVR and LVR rats were injected (i.p) with either saline vehicle or naltrexone (20 mg/kg) (Sigma) one hour into the dark-cycle (n = 6-7). NAc core, NAc shell, VTA, and Arc tissue were then collected from HVR and LVR rats 120-min post-injection. Previous studies have shown that naltrexone influences neuronal activation, as measured by Fos induction, 90-120-min post-injection (Dayas et al., 2007). Further, to minimize the effects of cycling hormones on cell activation, vaginal cytology was performed to ensure all rats were injected on the night of proestrus. Differences for Fos, dopamine receptors (Drd1, Drd2, and Drd5), and tyrosine hydroxylase (Th) mRNAs were then assessed by qRT-PCR.

Experiment 3

This experiment determined opioid antagonism effects on wheel running and food intake. Five-week-old HVR (n = 14) and LVR (n = 13) rats were given free wheel access for 3 weeks. At 8 weeks, each rat was injected (i.p.) with vehicle (sterile saline) or naltrexone (10, 20mg/kg) one hour into the dark cycle. Injections were counterbalanced so that all rats were given each treatment on the night of proestrus (3 estrous cycles), as determined by vaginal cytology. An interesting characteristic of females rats is their display of voluntary running distance varying with their 4-day estrous cycle (i.e., running distance peaks every 4^{th} night), with peak running occurring during proestrus (Anantharaman-Barr and Decombaz, 1989). Wheel running was monitored 120-min post-

injection. Food hopper weights were recorded at 2000 and 2200 h (immediately prior to, and 2 h post-injection, respectively).

Experiment 4

The effect of NAc dopaminergic neuron ablation on voluntary running was tested in HVR rats. Six week-old rats were introduced to running wheels. At 7 weeks, cannulae were bilaterally inserted to target the NAc shell. Following surgery, rats were injected with either 6-hyrdoxydopamine 6-OHDA (n = 9) to destroy dopaminergic nerve terminals or saline (n = 8). Given the high heterogeneity in running distances in the HVR line (unpublished observation), rats were matched-paired to vehicle or 6-OHDA according to running distance prior to surgery, with an additional rat assigned to the 6-OHDA group. Counter-balanced naltrexone injection occurred 21 days after surgery to ensure the loss of retrograde cells. Running distance and food intake were measured as in Experiment 3.

Tissue Extraction

During sacrifices, brains tissue was extracted using a 2mm-thick punch tool and brain sectioning apparatus (Braintree Scientific). Tissue plugs from 2mm-thick coronal brain slices were identified per a rat brain atlas (Paxinos and Watson, 1998).

Western Blotting

Western blotting was performed as previously described (Roberts et al., 2014). Twenty microgram of protein was loaded onto SDS-PAGE gels, transferred onto

nitrocellulose membranes, and incubated with Ponceau S (Sigma) to verify equal lane loading. Primary antibody for Oprm1 (1:500, Santa Cruz Biotechnology) was diluted in TBST with 5% BSA and applied to membranes overnight at 4°C. HRP-conjugated secondary antibody (1:1,000; Cell Signaling) was applied for 1 h at room temperature in TBST with 5% non-fat milk. Prior to exposure, ECL substrate (Pierce Biotechnology) was applied for 5 minutes. Band densitometry was obtained using a Kodak 4000R Imager and Molecular Imagery Software (Kodak Molecular Imaging Systems, New Haven, CT).

Tissue Dissociation and Electrophysiology

NAc punches were dissociated using a previously established procedure (Kuehl-Kovarik et al., 2002). Electrophysiological recordings from HVR and LVR NAc neurons (n = 16/line) occurred 16-24 h following dissociation, as previously described (Kuehl-Kovarik et al., 2002). Whole-cell recordings were obtained with thin-walled borosilicate glass micropipettes (World Precision Instruments), with a resistance of 2-3 MΩ, filled with a potassium gluconate intracellular solution [in millimoles: 120 potassium gluconate, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1 NaCl, 5 EGTA, 2 ATP, and 0.2 GTP (pH 7.2–7.4)]. The presence of 1 mM $CaCl_2$ during isolation and trituration prevents the retention of presynaptic boutons on dissociated neurons (Drewe et al., 1988) and ensures recordings from post-synaptic neurons. Recordings were performed using a MultiClamp 700B digitized with a Digidata 1440 and stored using pClamp 10 software (Molecular Devices).

Whole-cell current-clamp recordings were used to examine depolarizing or hyperpolarizing responses in cells isolated from HVR and LVR rats. The majority of cells

were firing (resting membrane potential <−50 to −55 mV) when whole-cell, current-clamp mode was achieved. Cells were hyperpolarized to −60 mV to prevent spontaneous activity. Given that medium spiny neurons (MSNs) represent 90-95% of neurons in the NAc (Yager et al., 2015), we assumed recordings were from MSNs, however characterizations of D1-type vs. D2-type MSNs were not made. MSNs were also identified by morphological analysis, as previously described (Calabresi et al., 1995; Calabresi et al., 1990). While recording in whole-cell current-clamp, each neuron was bathed in artificial cerebrospinal fluid (ACSF) (2 minutes), then ACSF + DAMGO (1 μM) for 4-6 minutes, and finally washed with ACSF (5-10 minutes). Previous studies have indicated that the EC50 for DAMGO in the NAc of the normal rat pup is 0.1 μM, suggesting that 1 μM DAMGO may be a saturating concentration (Chieng and Bekkers, 2001). Data were digitized at 20 kHz and low-pass filtered at 4 kHz. All amplitude measurements were taken in reference to the pre-pulse baseline. Latency and duration measurements were taken at the time of pulse initiation.

Each cell was analyzed as an individual data point. The amplitude of the response (depolarization or hyperpolarization) was determined in relationship to the baseline established before the application of DAMGO. Depolarization and hyperpolarization were treated as different responses and analyzed separately. Data were analyzed for 1) the percentage of cells that respond (with a >3 mV response) to DAMGO per rat line; and 2) the mean amplitude of response to DAMGO for each rat line.

Thermal Stimulation Test

Paw-withdrawal latency (PWL) to radiant heat was examined according to the Hargreaves test (Dirig et al., 1997; Hargreaves et al., 1988) using a Model 390 Paw Stimulator Analgesia Meter (IITC/Life Science Instruments; Woodland Hills, CA). This test was performed to assess potential differences in peripheral opioid signaling. Rats were individually placed into Plexiglas chambers on an elevated glass floor and habituated to the environment for 30 minutes. A beam of radiant heat at low (10%) stimulus intensity was applied to the plantar surface of each hind paw through the glass plate. When the rat lifted its foot, the light beam was turned off. The length of time between the start of the light beam and the foot lift was measured by the apparatus and defined as the PWL. A cut-off time of 20 seconds was used to avoid tissue damage to the paw and there was a 5-minute interval between testing left and right paws. The trials were then repeated for increasing (20%, 40%, 60%, 80% and 100%) intensities with 5-minute intervals between intensities.

RNA isolation, cDNA synthesis, and qRT-PCR

RNA isolation, cDNA synthesis, and qRT-PCR were performed as previously described (Ruegsegger et al., 2015). Gene-specific primers were constructed using PrimerExpress3.0 (Applied Biosystems) (Table 2.1). Twenty nanograms of cDNA from each sample were assayed in duplicate using SYBR Green Mastermix (Applied Biosystems). mRNA expression values were quantified using the $2^{\Delta\Delta Ct}$ method, whereby ΔCT = 18S Ct – target gene Ct.

Table 2.1. Primer sequences for gene expression analyzed by qRT-PCR

Gene	Forward (5'- 3')	Reverse (5'- 3')
18S	GCCGCTAGAGGTGAAATTCTTG	CATTCTTGGCAAATGCTTTCG
Fos	GGAGCCGGTCAAGAACATTA	ATGATGCCGGAAACAAGAAG
Drd1	TCTCCTGGGCAATACCCTTGT	GGACCTCAGGTGTCGAAACC
Drd2	GTCCTGGTACGATGACGATCTG	CCTTCCCTTCTGACCCATTG
Drd5	CAACTCAATTGGCACAGAGACAA	TTGGACAGCAGGCCCTCTT
Th	TGTTGGCTGACCGCACAT	CCCAGAGATGCAAGTCCAATG

Surgery and 6-OHDA administration

Brain cannula were implanted as previously described (Ruegsegger et al., 2015). On the day of surgery, rats (165-215 g) were anesthetized with a mixture of ketamine/xylazine (87/13 mg/kg). Cannulae were bilaterally implanted 2.5 mm above the NAc shell using coordinates (relative to Bregma): 1.30 mm rostral, 0.80 mm from midline, 6.40 mm ventral. Infusions were performed using a 31-gauge injector attached to polyethylene tubing and 10-mm Hamilton syringes mounted to an infusion pump (Harvard Apparatus). 6-OHDA hydrobromide (12 μg/0.5 μl) (Sigma) or vehicle (0.2% ascorbic acid) were bilaterally infused over 2 min for a final volume of 0.5 μl. Injectors remained in place for 2 additional minutes to allow proper infusion. 30-gauge stylets were inserted into the guide cannulae to prevent occlusions. To spare noradrenergic terminals, desipramine (15 mg/2 ml/kg i.p.) was injected 30-min before 6-OHDA injections. Following initial recovery, rats were placed back into their home cages with running wheels and monitored for 21 days before beginning naltrexone or saline i.p. injections.

Immunohistochemistry for 6-OHDA dopamine neuron lesion

6-OHDA depletion of dopamine neurons was assessed by tyrosine hydroxylase immunostaining (1:1000, EMD-Millipore), as previously described (Roberts et al., 2014). Micrographs were captured using an Olympus BX60 photomicroscope (Olympus), and photographed with a Spot Insight digital camera (Diagnostic Instruments). TH-positive neurons in the NAc and dorsal striatum were counted using Fiji (ImageJ) software (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Data were analyzed with SigmaPlot 12.0 (Systat Software, Inc). Significance was set with an alpha-value of 0.05. Student's t-test compared differences in Oprm1 mRNA, protein, and electrophysiological responses following DAMGO application between HVR and LVR rats. Differences in Oprm1 mRNA expression between brain regions was assessed by a one-way analysis of variance (ANOVA). For thermal stimulation, the average of the PWL was log transformed and analyzed via two-way ANOVA. Two-way ANOVA was used to assess the influences of naltrexone on mRNA expression. Repeated-measures ANOVA assessed incremental differences in running distance following the dose-dependent injection of naltrexone in Experiments 3 and 4. Repeated-measures ANOVA compared differences in food intake in Experiments 3 and 4. Basal differences in running distance in Experiment 4 were assessed with paired-t-test. Holm-Sidak analysis was used for all post-hoc analysis when appropriate.

RESULTS

Experiment 1

Oprm1 mRNA was increased in the NAc (core and shell) ($p = 0.014$) and Arc ($p = 0.025$) of HVR compared to LVR rats (Figure 2.1A). Additionally, one-way ANOVA inclusive of both HVR and LVR showed that Oprm1 mRNA expression was influenced by brain region ($F_{5,78} = 36.2$, $p < 0.001$) (Figure 2.1B). Oprm1 protein in the NAc was ~2-fold greater in HVR rats ($p = 0.020$) (Figure 2.1C). Two-way ANOVA found that Oprm1 protein was increased in the Hb compared to ECN ($F_{1,12} = 74.2$, $p < 0.001$), while Oprm1 protein in the Hb ($p = 0.15$) or ECN ($p = 0.96$) was not different between HVR or LVR (Figure 2.1D). Whole-cell current-clamp recordings from NAc neurons showed no differences in capacitance (Cm) (HVR: 9.34 ± 0.68, LVR: 9.78 ± 1.23, $p = 0.77$) between isolated HVR and LVR NAc neurons (Figure 2.1E). Following administration of 1μM DAMGO, the mean average response (mV) for all neurons trended ($p = 0.060$) to be increased in HVR neurons (HVR: 4.82 ± 2.26, LVR: -0.13 ± 1.14) (Figure 2.1F). Additionally, ~45% of HVR neurons (7/16) produced depolarizing responses (>3mV response), compared to 25% of LVR neurons (4/16) depolarizing following DAMGO application (Figure 2.1G). Of depolarizing cells, the mean average response was greater in HVR than LVR NAc neurons (HVR: 13.08 ± 2.50, LVR: 4.90 ± 1.21, $p = 0.044$) (Figure 2.1H). Together, these results suggest Oprm1 function is inherently greater in the NAc of HVR compared to LVR rats.

No significant differences between HVR and LVR rats in the PWL responses were present across stimulus intensities ($F_{2,130} = 1.23$, $p = 0.30$) (Figure 2.2).

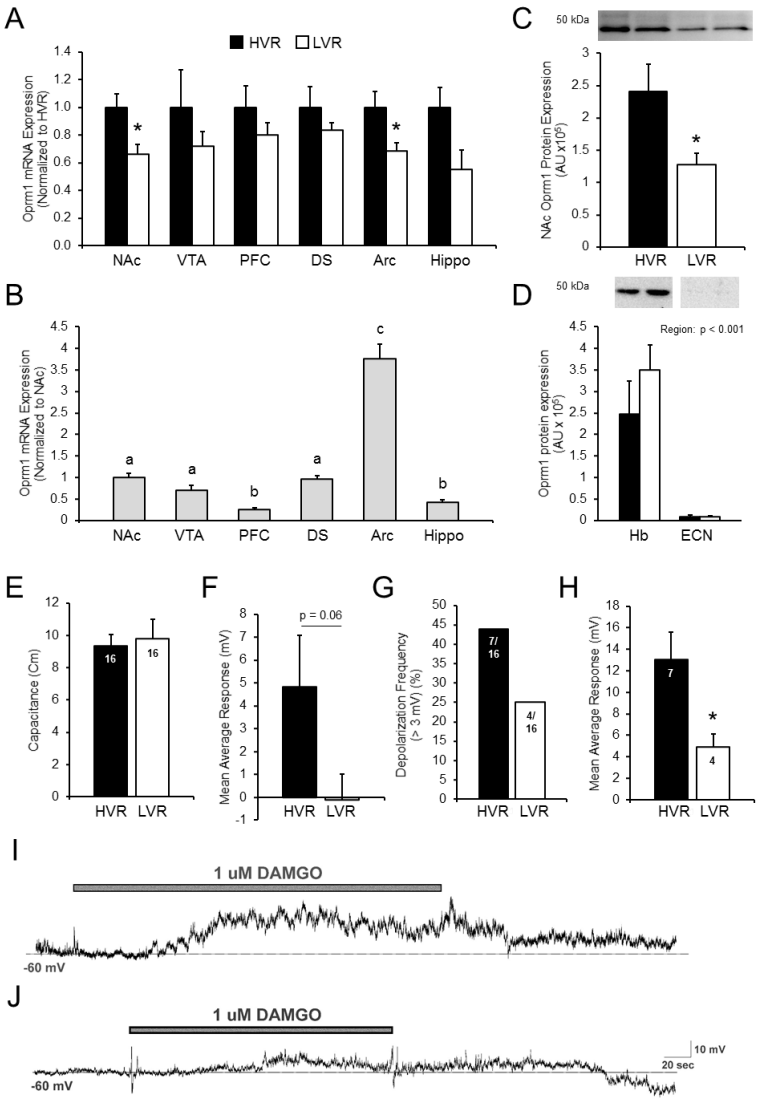

Figure 2.1. Oprm1 expression and functions differ between HVR and LVR. Oprm1 mRNA is increased in the NAc (core and shell) and arcuate nucleus (Arc) in sedentary HVR, versus LVR, rats (A). In analysis inclusive of both HVR and LVR rats, Oprm1 mRNA was differentially expressed depending on brain region [note: different letters denote different statistical significance ($p < 0.05$)] (B). Oprm1 protein is greater in the NAc of HVR rats (C), and increased in the Hb compared to ECN in both HVR and LVR rats (D). We performed current-clamp electrophysiology on isolated NAc neurons from HVR and LVR rats (n = 16/line) (E). For all neurons analyzed, HVR neurons tended ($p = 0.06$) to have a greater mean average response (mV) to 1μM DAMGO treatment (F). Application of DAMGO produced depolarizing responses (> 3 mV) in 44% (7/16) and 25% (4/16) of HVR and LVR neurons, respectively (G). Of the neurons depolarized following DAMGO application, the mean average response was 2.7-fold greater in HVR neurons (H). Representative traces for HVR (I) and LVR (J) neurons. Scale bar in (J) also applies to (I). Values are presented as mean ± SEM. * indicates significant difference between HVR and LVR ($p < 0.05$).

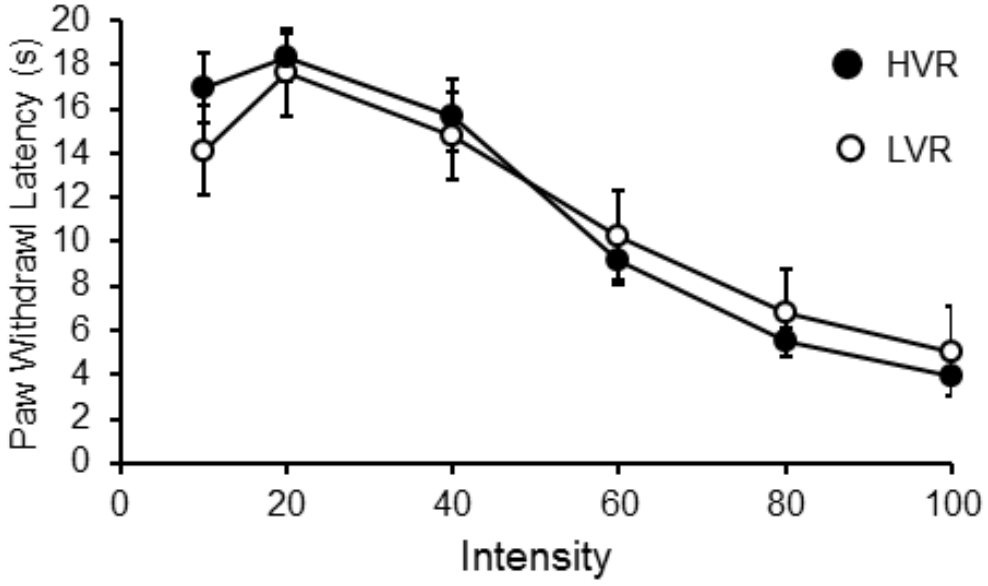

Figure 2.2. Paw withdrawal latency is similar between HVR and LVR. Paw latency withdrawal in seconds at varied thermal intensities for HVR (closed circles) and LVR (open circles) rats (n = 11-12/line). Values are presented as mean ± SEM.

Experiment 2

All qRT-PCR data are shown in Table 2.2. Two-way ANOVA of mRNA expression in the NAc core showed that naltrexone reduced Fos mRNA expression ($F_{1,22}$ = 8.06, p = 0.010), while Drd2 ($F_{1,22}$ = 4.57, p = 0.044) and Drd5 ($F_{1,22}$ = 8.09, p < 0.01) mRNA were higher in HVR compared to LVR rats. Analysis of mRNA expression in the NAc shell showed that naltrexone significantly reduced Fos ($F_{1,22}$ = 6.54, p = 0.018) and Drd5 ($F_{1,22}$ = 7.87, p = 0.010) mRNA expression. Post-hoc analysis revealed that naltrexone reduced Fos (p = 0.015) and Drd5 (p = 0.035) mRNA expression in HVR rats, but had no effect in LVR rats (Figure 2.3A, C). Analysis of mRNA expression in the Arc revealed that naltrexone reduced Fos mRNA expression ($F_{1,22}$ = 5.66, p = 0.026), with post-hoc analysis determining Fos mRNA decreased following naltrexone in HVR rats (p = 0.014). Analysis of mRNA expression in the VTA identified a line x drug interaction ($F_{1,22}$ = 5.97, p = 0.023) for Th mRNA expression, with post-hoc analysis showing

naltrexone reduced Th mRNA expression in HVR ($p = 0.031$) but not LVR rats (Figure 2.3B, D), and Th mRNA was increased in HVR compared to LVR rats treated with saline ($p = 0.028$).

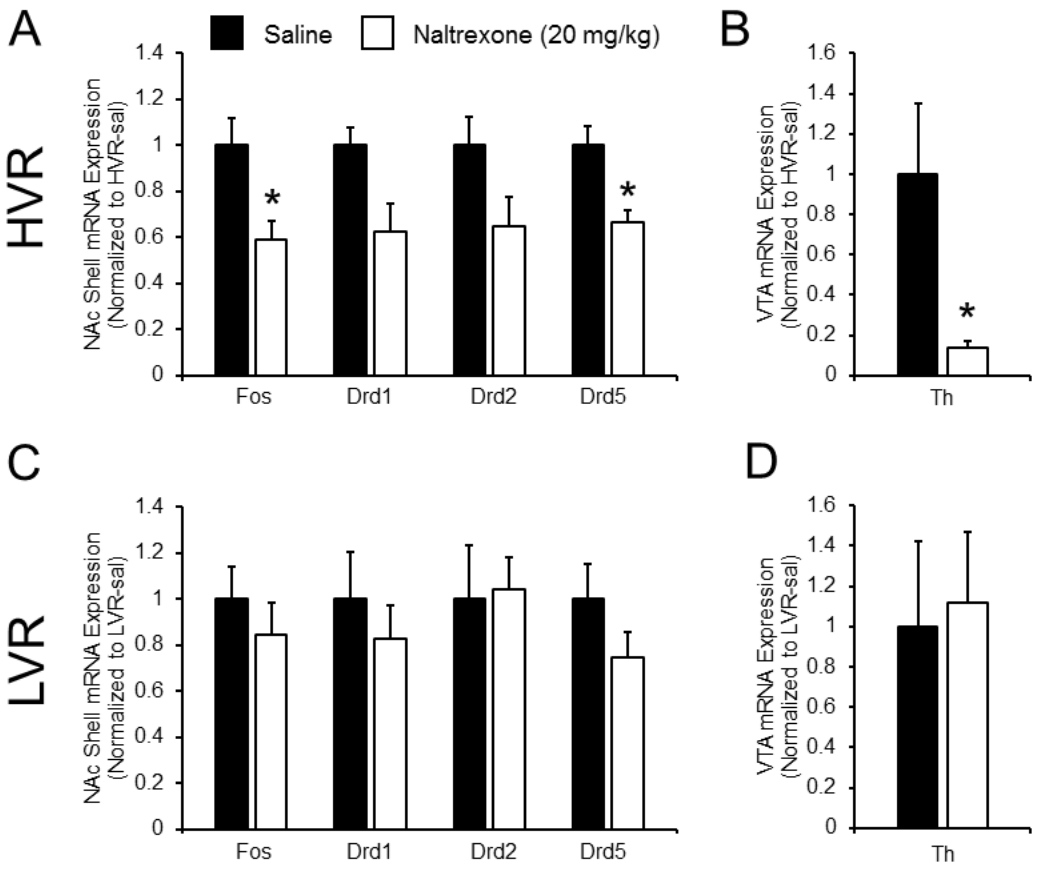

Figure 2.3. NAc and VTA mRNA expression following naltrexone injection. Relative mRNA expression of Fos, Drd1, Drd2, Drd5, and Th 120-min post i.p. saline (black) or naltrexone (20 mg/kg) (white) injection for the nucleus accumbens shell (A) and ventral tegmental area (B) of HVR rats and for the nucleus accumbens shell (C) and ventral tegmental area (D) of LVR rats. Values are presented as mean ± SEM (n = 6-7/group). * indicates significant difference between saline and naltrexone ($p < 0.05$).

Table 2.2. Full qRT-PCR results following naltrexone injection

	HVR-sal	HVR-ntx	LVR-sal	LVR-ntx
NAc core				
Fos†	1.00 ± 0.10	0.69 ± 0.09	0.90 ± 0.13	0.57 ± 0.12
Drd1	1.00 ± 0.13	0.87 ± 0.14	0.78 ± 0.12	0.60 ± 0.07
Drd2*	1.00 ± 0.09	0.95 ± 0.09	0.72 ± 0.12	0.69 ± 0.06
Drd5*	1.00 ± 0.09	0.97 ± 0.17	0.78 ± 0.12	0.52 ± 0.09
NAc shell				
Fos†	1.00 ± 0.13	0.58 ± 0.09‡	0.85 ± 0.12	0.71 ± 0.12
Drd1	1.00 ± 0.09	0.62 ± 0.13	0.73 ± 0.15	0.61 ± 0.10
Drd2	1.00 ± 0.12	0.64 ± 0.13	0.90 ± 0.21	0.94 ± 0.12
Drd5†	1.00 ± 0.08	0.67 ± 0.05‡	0.74 ± 0.11	0.56 ± 0.08
Arcuate nucleus				
Fos†	1.00 ± 0.08	0.76 ± 0.06‡	0.78 ± 0.07	0.73 ± 0.06
Drd1	1.00 ± 0.12	0.85 ± 0.10	0.85 ± 0.07	1.09 ± 0.13
Drd2	1.00 ± 0.09	0.90 ± 0.09	0.80 ± 0.12	0.80 ± 0.11
Drd5	1.00 ± 0.04	0.82 ± 0.06*	0.77 ± 0.07	0.75 ± 0.06
VTA				
Th#	1.00 ± 0.35	0.14 ± 0.03‡	0.33 ± 0.14‡	0.37 ± 0.12

Values are mean ± SEM. mRNA expression values are presented as $2^{\Delta\Delta CT}$ whereby ΔCT = 18S CT − gene of interest CT. Expression values were normalized to 1.0 for HVR-sal. Statistical comparisons were made with two-way ANOVA. Symbols: * denotes significant line (HVR vs. LVR) effect, † denotes significant drug (saline (sal) vs. naltrexone (ntx), # denotes significant line x drug interaction, ‡ denotes significant statistical difference from HVR-sal ($p<0.05$) (n = 6-7/group).

Experiment 3

Running patterns and food intake following injection of either saline or 10 or 20 mg/kg naltrexone are shown in Figure 2.4. Analysis of running and feeding behavior on the night of saline injection with one-way ANOVA determined that HVR rats ran ($F_{1,25} = 77.69$, $p < 0.001$) significantly more than LVR rats, however no differences in food intake were present ($F_{1,25} = 3.37$, $p = 0.079$). Repeated-measures ANOVA showed a significant effect of naltrexone administration on running distance in HVR ($F_{2,76} = 12.59$, $p < 0.001$), but not LVR rats ($F_{2,72} = 1.89$, $p = 0.17$). Post-hoc analysis revealed wheel running was decreased in HVR rats following both 10 and 20 mg/kg naltrexone administration compared to saline ($p < 0.05$). Repeated-measures ANOVA showed that

naltrexone significantly decreased food intake in HVR ($F_{2,18} = 7.90$, $p < 0.01$), but not LVR ($F_{2,23} = 0.79$, $p = 0.51$) rats. Post-hoc analysis revealed that both 10 ($p = 0.01$) and 20 mg/kg ($p < 0.01$) doses of naltrexone decreased food intake, compared to saline, in the HVR line.

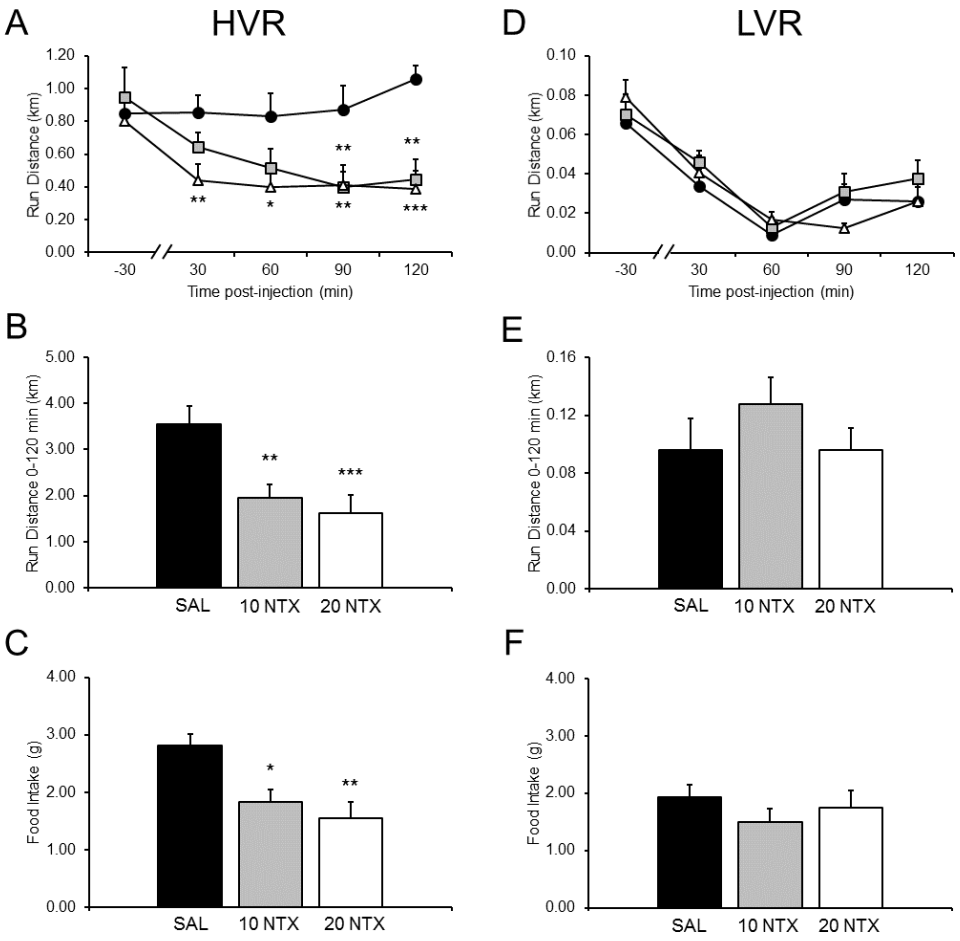

Figure 2.4. Wheel running following systemic naltrexone injection. Wheel running and food intake responses following intraperitoneal injection of saline (SAL) (black circle), 10 mg/kg naltrexone (NTX) (gray square), or 20 mg/kg NTX (open triangle). Panels A-C depict running distance in 30-min segments, running distance for the entire 120-min test session, and food intake for the entire 120-min test session in HVR rats, respectively. Panels D-F depict running distance in 30-min segments, running distance for the entire 120-min test session, and food intake for the entire 120-min test session in LVR rats, respectively. Values are presented as mean ± SEM (n = 13-14/group). Symbols indicate significant difference from saline: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Experiment 4

6-OHDA infusion reduced TH-immunoreactivity 81% and 43% in the NAc and dorsal striatum, respectively, compared to saline injection (Figure 2.5A). No relationships between the size or location of the lesion and running distance or food intake were observed (data not shown). Paired t-test showed 6-OHDA decreased wheel running in the 1st week following infusion ($p < 0.01$) (Figure 2.5B).

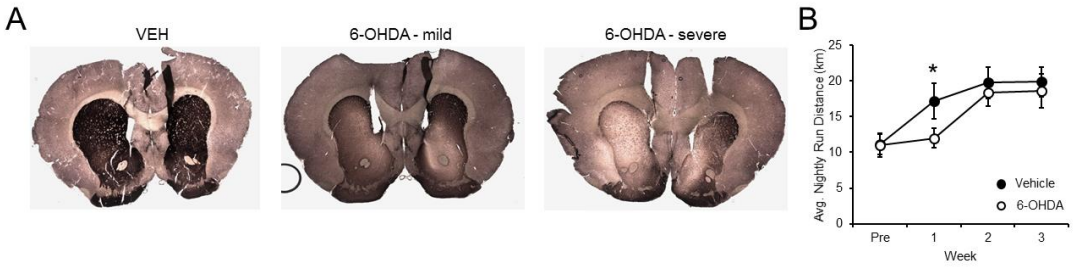

Figure 2.5. 6-OHDA influence on wheel running. Representative tyrosine hydroxylase (TH) stained coronal sections of the striatum from rats that received either vehicle (VEH) (left panel), or 6-hydroxydopamine (6-OHDA). 6-OHDA induced lesions ranged from mild (middle panel) to severe (right panel). 6-OHDA depletion of TH immunostaining was noted in the NAc and dorsal striatum (A). Paired t-test showed that 6-OHDA (open circles) infusion into the NAc decreased wheel running in the initial week following injection (B). Values are presented as mean ± SEM. * indicates significant difference between VEH and 6-OHDA ($p < 0.05$).

Running patterns and food intake following injection of either saline or 5, 10 or 20 mg/kg naltrexone in vehicle or 6-OHDA treated rats are shown in Figure 2.6. Analysis of running and feeding behavior on the night of saline injection with one-way ANOVA found no differences in running distance ($F_{1,15} = 1.26$, $p = 0.280$) or food intake ($F_{1,25} = 1.22$, $p = 0.287$) between vehicle and 6-OHDA injected rats. Repeated-measures ANOVA showed a significant effect of naltrexone on running distance in vehicle ($F_{3,63} =$

11.158, $p < 0.001$), but not 6-OHDA-injected HVR rats ($F_{3,72} = 1.068$, $p = 0.381$). Post-hoc analysis revealed wheel running was decreased in vehicle-treated HVR rats following 10 and 20mg/kg naltrexone administration ($p < 0.05$). For the entire 120-min experimental session, repeated-measures ANOVA showed that naltrexone significantly decreased food intake in both vehicle ($F_{3,20} = 4.96$, $p < 0.01$) and 6-OHDA injected HVR rats ($F_{3,24} = 9.732$, $p < 0.001$). Post-hoc analysis revealed that 20 mg/kg ($p < 0.01$) and 10 and 20 mg/kg ($p < 0.001$) naltrexone injections decreased food intake in vehicle and 6-OHDA-treated HVR rats, respectively.

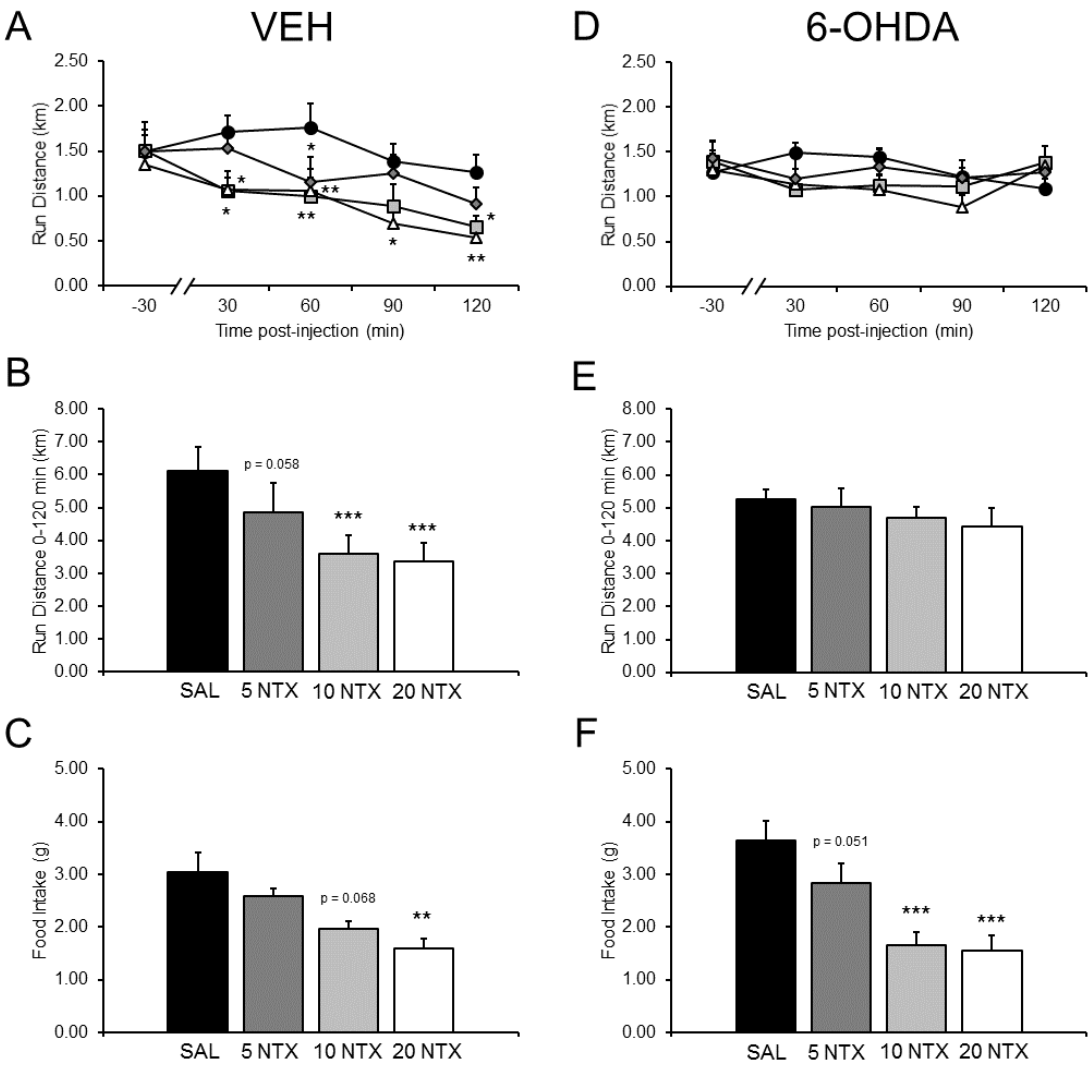

Figure 2.6. 6-OHDA influence on naltrexone-induced changes in wheel running. Wheel running and food intake responses following intraperitoneal injection of saline (SAL) (black circle), 5 mg/kg naltrexone (NTX) (dark gray diamond), 10 mg/kg NTX (gray square) or 20 mg/kg NTX (open triangle) in HVR rats. Panels A-C depict running distance in 30-min segments, running distance for the entire 120-min test session, and food intake for the entire 120-min test session in vehicle (VEH) treated rats, respectively. Panels D-F depict running distance in 30-min segments, running distance for the entire 120-min test session, and food intake for the entire 120-min test session in 6-hydroxydopamine (6-OHDA) treated rats, respectively. Values are presented as mean ± SEM (n = 8-9/group). Symbols indicate significant difference from saline: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

To our knowledge, this is the first report to analyze how opioidergic and dopaminergic signaling work together to mediate wheel running behavior. Given the polygenic nature of human physical activity (Lippi et al., 2008), our unique model of high and low voluntary running provides a valuable tool to understand running neurobiology, and potentially why a large proportion of the U.S. population is not meeting physical activity guidelines (Troiano et al., 2008).We report the following novel findings: 1) Oprm1 expression and function are higher in the NAc of sedentary HVR than LVR rats, 2) blocking opioid-receptor function with naltrexone decreases mRNAs related to dopamine signaling in HVR, but not LVR, rats, 3) naltrexone reduces wheel running and food intake in HVR, but not LVR rats, and 4) HVR rats with ablated NAc dopaminergic neurons are refractory to naltrexone-induced reductions in wheel running, but not food intake. Together, these findings suggest that inherent differences in Oprm1 action and downstream dopaminergic signaling may influence inherited physical activity behavior. Collectively with previous studies (Brown et al., 2015; Roberts et al., 2012; Roberts et al., 2014; Ruegsegger et al., 2015), a model by which inherent differences in the mesolimbic dopamine system may contribute to differing physical activity levels between HVR and LVR rats is presented in Figure 2.7.

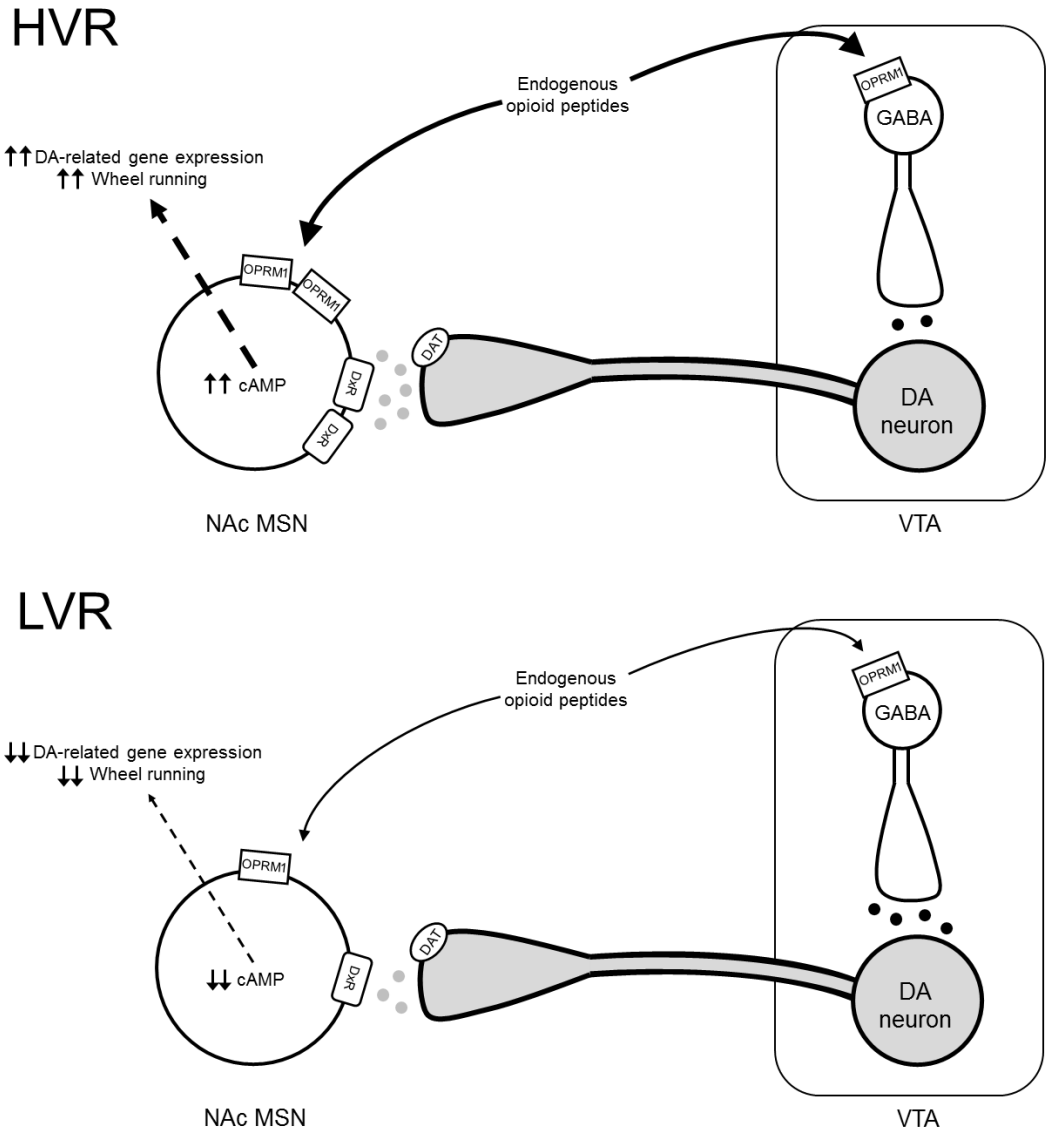

Figure 2.7. Hypothesized, collective model summarizing how inherent differences in the mesolimbic dopamine system may influence physical activity levels in HVR and LVR rats. Previous evidence suggests endogenous opioids are increased in HVR compared to LVR rats. Despite no difference in Oprm1 expression in the VTA, we speculate elevated endogenous opioids may lead to inherent increases in DA release in the NAc of HVR rats indirectly via disinhibition of GABAergic neurons in the VTA. The effect of DA in HVR rats on down-stream cAMP production and DA-related gene transcription may be further amplified by increased dopamine receptor expression in the NAc, together with similar levels of DAT, compared to LVR rats. Increased Oprm1 expression in the NAc of HVR rats may enhance DA-related gene transcription. In total, we speculate these adaptations may lead to the drastic divergence in wheel running between HVR and LVR rats. Abbreviations: DA: dopamine; DxR: dopamine receptor; DAT: dopamine transporter; MSN: medium spiny neuron; OPRM1: mu opioid receptor 1.

At least some of the rewarding effects of physical activity are caused by the stimulation of the same brain circuits that are activated by drugs of abuse, including opioidergic and dopaminergic systems (Knab and Lightfoot, 2010). In the current study, we focused on opioidergic signaling due to our observations that HVR rats express elevated amounts of Oprm1 mRNA and protein in the NAc (core and shell) compared to LVR rats, and because Oprm1 expression is associated with various rewarding behaviors (Mansour et al., 1995). We demonstrated that NAc neurons from HVR rats are more sensitive to mu-opioid receptor-stimulated depolarization than NAc neurons isolated from LVR rats. While opioid receptors often work by disinhibition, Ma et al. (Ma et al., 2012) demonstrated that DAMGO induces depolarizations in D1 cells of the NAc and hyperpolarized the threshold for action potential generation in D2 cells of the NAc, indicating increased excitability of NAc medium spiny neurons (MSN)s by DAMGO. Given that Oprm1 is primarily expressed by dynorphin- and D1-expressing cells (Georges et al., 1999), we posit that greater activation of the striatonigral pathway could lead to disinhibition of motor circuits that facilitate the motivation for voluntary running in HVR rats. However, the current lack of D1 vs. D2 MSN comparisons and recording Oprm1 responses only on the postsynaptic membrane limit our interpretation. For example, our results could represent a difference in the proportion of Opm1 expressing (direct-pathway) neurons in each group. Additionally, these results could be the effect of DAMGO indirectly effecting dopamine terminals or off-target effects of DAMGO. Nonetheless, our results imply heightened Oprm1 action in the NAc of HVR compared to LVR rats.

Pharmacological and transgenic animal studies show that Oprm1 is important for responses to thermal pain (Chen and Pan, 2006; Gendron et al., 2007). Interestingly, our data show that although HVR rats express higher levels of Oprm1 than LVR rats, we did not observe differences in sensitivity to thermal pain between HVR and LVR rats. These findings differ with those from rats selectively bred for high and low forced treadmill running distance (Geisser et al., 2008). This comparison suggests that pain sensitivity may carry a stronger co-selected genetic inheritance in selective breeding models based on forced-exercise rather than voluntary running behavior.

Our results suggest that HVR rats may have elevated baseline opioidergic activity compared to LVR rats and may be hypersensitive to endogenous opioid action. Naltrexone reduces ethanol-induced increases in Th mRNA to baseline levels (Lee et al., 2005; Navarrete et al., 2014), an effect which may contribute to an increase in dopamine synthesis. However, we did not observe Oprm1 mRNA differences in the VTA of HVR compared to LVR rats, suggesting other factors could influence Oprm1 function in HVR rats. Further, a prior report shows no influence of naltrexone on Fos mRNA expression in the NAc of naïve rats (Rasmussen et al., 1995); however, naltrexone does increase Fos mRNA in morphine-dependent rats (Georges et al., 2000; Rasmussen et al., 1995). Thus, naltrexone-induced decreases in Th and Fos mRNA expression in the VTA and NAc shell of HVR rats suggests that they may have an elevated basal level of opioidergic signaling similar to those in opioid-related addiction phenotypes. However, these data have several limitations. Home cage activity was not measured in the current study, thus it is possible that diverging levels of home cage activity, rather than inherent genetic differences, influence the observed results. Given the high dose of naltrexone (20 mg/kg)

used in this experiment, the observed changes in mRNA expression may also be influenced by kappa and delta opioid receptor activity. Future studies using low-dose subcutaneous (s.c.), rather than i.p., naltrexone injection should be performed given the ~30-times more potent effect of naltrexone with s.c. injection (Williams and Broadbridge, 2009).

We demonstrate that opioidergic inhibition modifies dopaminergic signaling in HVR, but not LVR, rats. Opioidergic modification of dopamine action is well described (Mansour et al., 1995). Several reports show that opioid-receptor antagonism suppresses ethanol-induced dopamine-release in the NAc, a behavior well characterized to involve Oprm1 in the NAc (Benjamin et al., 1993; Gonzales and Weiss, 1998). Additionally, Oprm1 influences other dopamine-dependent processes such as cocaine self-administration (Mathon et al., 2005). Likewise, our results suggest that naltrexone influences expression of neurons expressing D1-like receptors, which is in agreement with the notion that Oprm1 is primarily expressed on D1-like neurons (Georges et al., 1999). Thus, blocking endogenous opioid action in HVR rats likely reduces contributions from neurons in striatonigral "direct" pathway, which express dynoprphin and D1-like dopamine receptors, for the promotion of rewarding behavior. We previously noted that dynorphin mRNA is intrinsically increased in the NAc of HVR rats versus LVR rats (Ruegsegger et al., 2015). Further, our findings show that naltrexone influences dopamine receptor expression in the NAc shell but not NAc core. In contrast, a previous finding suggests the NAc core, and not shell, likely mediates voluntary running behavior (Werme et al., 2002). Future research is needed to clarify the specific contributions of the NAc core and shell to wheel running behavior.

We demonstrate that opioid antagonism decreases wheel-running behavior in HVR rats. These results are in agreement with previous rat (Li et al., 2004; Sisti and Lewis, 2001) and hamster (Schnur and Barela, 1984) studies. In contrast, voluntary running of LVR rats is not influenced by opioid antagonism. We posit that the inability of LVR rats to respond to naltrexone is a result of lower opioid receptor expression and, therefore, depressed opioid signaling. This may at least partially account for the lower volume of running exhibited by LVR rats compared to HVR and outbred rats. However, we did not observe differences in fecal excretion between HVR and LVR rats following naltrexone injection suggesting naltrexone did not change basal levels of endogenous opioids (observer observation). Given changes in gene expression in the VTA and NAc shell following naltrexone injection, we predict that the NAc and VTA may be the sites of action where systemic naltrexone injection influences wheel running behavior in HVR rats. Further, while naltrexone did not impact running in LVR rats, the possibility of a "floor effect" not allowing a decline in running given the low basal running distance of LVR rats must remain viable and still be considered.

We observed a reduction in running of HVR rats following 6-OHDA infusion into the NAc shell. This suggests the mesolimbic dopamine system partially contributes to the heightened wheel running performed by HVR rats. However, as the effect disappeared two weeks post-lesion, it is possible that neuro-adaptive changes may have taken place to compensate for the disrupted dopaminergic system and thus masked the effects of dopaminergic lesion on wheel running. However, the above results of the initial decreases in wheel running are in agreement with Robbins & Koob (Robbins and Koob, 1980), who demonstrated that 6-OHDA lesion in the NAc decreases voluntary wheel running. In the

present study, catecholamine loss in the dorsal striatum following 6-OHDA infusion may have also contributed to the acute decrease in wheel running in HVR rats. Dopamine concentrations in the dorsal striatum are increased in mice bred for high voluntary wheel running (Mathes et al., 2010), and ΔFosB in striatal neurons influences wheel running (Werme et al., 2002).

Given that dopamine depletion produced a small change in running, these results also suggest that dopamine is important for the motivational, but not locomotor, components of wheel running. Thus wheel running may not be a direct index of physical activity motivation. Nevertheless, many studies have associated the mesolimbic dopamine system with the motivation for voluntary wheel running [see (Knab and Lightfoot, 2010) for additional refs]. The finding that 6-OHDA transiently decreases running in HVR rats also extends upon several previous studies by our lab showing the HVR rats are sensitive to both Drd1 agonism and antagonism in the NAc compared to LVR rats (Roberts et al., 2012). Therefore, we speculate that an inherent increased potential to respond to dopamine may drive the high running phenotype in the HVR line. Given the response of HVR rats to 6-OHDA with current and past data showing LVR rats are unresponsive to opioid or dopamine modulation, we posit that LVR rats are insensitive to changes in dopamine, potentially due to an inherent reduction in MSNs and immature neuron number in the NAc compared to HVR rats (Roberts et al., 2014). However, previous unpublished observations from G12 HVR and LVR rats found no difference in TH-immunoreactivity, suggesting dopamine projections to the NAc are unchanged between HVR and LVR rats (unpublished data). Additionally, the potential

ablation of noradrenergic neurons by 6-OHDA may have also contributed to the phenotypes observed in the current study.

Ablation of NAc shell dopaminergic neurons by 6-OHDA blocked naltrexone-induced decreases in voluntary running by HVR rats. This observation suggests that naltrexone influences wheel running by blocking the natural reward from running rather than other mechanisms which dictate physical activity reward which could include reducing pain perception during exercise (Tierney et al., 1991), β-endorphin-mediated glucose uptake in skeletal muscle (Evans et al., 1997), or by inhibiting the ability of β-endorphin to delay fatigue (Khan and Smith, 1995). Given these results, we speculate an interaction between the opioidergic and dopaminergic systems in the NAc may be critical for perceiving reward from physical activity. However, given the far-reaching effect of 6-OHDA on retrograde neurons, it is possible other brain regions may mediate this interaction as well.

The findings of this study have important implications for dissecting out the unique neural-circuitry that drive energy intake and physical activity-associated energy expenditure. 6-OHDA infusion in the NAc shell did not block naltrexone-induced decreases in food intake by HVR rats. This suggests that opioids may influence wheel running and free-feeding behavior in dopamine-dependent and independent mechanisms, respectively. This observation is in agreement with our previous study following intra-NAc infusion of DAMGO which showed that wheel running behavior was altered independent of feeding behavior, and together these results suggest the localized site of action for wheel running in the current experiment may be the NAc (Ruegsegger et al., 2015). Additional studies support a disconnect between dopaminergic and opioidergic

action for controlling food intake. For example, pretreatment with dopamine D1 and D2 antagonists does not blunt free feeding following DAMGO infusion into the NAc (Will et al., 2006). Additionally, dopamine is a strong mediator of tasks requiring varying degrees of effort and motivation to seek reward, such as voluntary wheel running, rather than free feeding behavior (Baldo and Kelley, 2007). Taken together, these intriguing findings imply opioidergic influences on wheel running behavior require dopamine transmission, and changes in effort and motivation for reward, while opioidergic regulation of free feeding may not.

Given the pandemic rates of physical inactivity and its associated comorbidities, understanding the molecular mechanisms that control physical activity is of great importance. These novel results suggest inherent reductions in Oprm1 signaling may contribute to lower physical activity. Similarly, mesolimbic dopamine signaling is essential to opioid-reward for voluntary exercise, but not for food intake. These data encourage future investigation into leveraging opioidergic and dopaminergic systems together to elevate physical activity in sedentary individuals.

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CHAPTER 3: Maternal Western diet promotes age-specific alterations in female offspring voluntary wheel running

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ABSTRACT

Prenatal overnutrition affects development into adulthood and influences obesity risk. Given the increasing prevalence of obesity during pregnancy, we assessed the transgenerational effect of maternal Western diet (WD) consumption on offspring physical activity. Voluntary wheel running was increased in juvenile (4-7 weeks of age), but decreased in adult (16-19 weeks of age), F_1 female WD offspring. In contrast, no wheel running differences in F_1 male offspring were observed. Intriguingly, increased voluntary running in juvenile, female, WD offspring associated with up-regulated Drd1 and Drd2 in the nucleus accumbens (NAc) and with down-regulated Lepr in the ventral tegmental area (VTA). Conversely, decreased voluntary running by adult, female, WD offspring associated with down-regulated Drd1 in the NAc and with up-regulated Lepr in the VTA. Surprisingly, body fat, leptin, and insulin were increased in male, but not female, F_1 WD offspring, suggesting changes in wheel running in female WD offspring were independent of metabolic disease risk. However, wheel running attenuated metabolic changes in male WD offspring. Analysis of F_2 offspring found no differences in wheel running or adiposity in male or female offspring, suggesting changes in the F_1 generation were likely due in utero somatic reprogramming rather than stable germline inheritance. Our findings indicate prenatal WD exposure leads to age-specific changes in voluntary physical activity in female offspring that are associated with changes in dopamine and leptin signaling in mesolimbic brain nuclei.

INTRODUCTION

The incidence of obesity during pregnancy, which ranges from 20-38% (Yogev and Catalano, 2009), increases the risk for obesity and metabolic diseases in both child (Mehta et al., 2012) and adult (Lawlor et al., 2011) offspring. In utero overnutrition exposure is postulated to influence physical activity levels, an important preventer of at least 35 chronic diseases (Booth et al., 2012). Rowland (Rowland, 1998) proposed that an "activity-stat" may be influenced by environmental factors during critical pre- and post-natal periods to regulate the propensity for physical activity. For example, a small-litter mouse model of early postnatal overnutrition reduces spontaneous activity and energy expenditure in adult females (Li et al., 2013). Paradoxically, both maternal food restriction and high-fat feeding in rats also decrease offspring locomotor activity in adulthood (Cunha Fda et al., 2015; Vickers et al., 2003). While these findings collectively suggest that excess nutritional status during pregnancy detrimentally affects offspring physical activity, the mechanisms remain largely unstudied. Specifically, understanding how maternal overnutrition influences voluntary physical activity (e.g. wheel running) is paramount given the often profound impact of exercise on fat loss (Garland et al., 2011).

One mechanism by which maternal overnutrition could influence offspring physical activity levels is via dysregulation of mesolimbic dopaminergic pathway, which at least partially regulates wheel-running (Knab and Lightfoot, 2010). Several reports demonstrate that maternal high-fat feeding alters dopamine-related mRNA expression and DNA methylation patterns in the nucleus accumbens (NAc) and ventral tegmental area (VTA) (Vucetic et al., 2011; Vucetic et al., 2010). Furthermore, expression patterns of dopamine and opioid receptors and ligands are up-regulated in juvenile, but down-

regulated in adult, offspring of "junk food" fed dams (Ong and Muhlhausler, 2011). Moreover, recent evidence from dopamine receptor 2 (Drd2) knockdown mice suggests decreased mesolimbic dopamine function reduces physical activity, in turn promoting obesity (Beeler et al., 2016).

Leptin receptor signaling is well characterized in VTA dopaminergic neurons (Domingos et al., 2011; Fulton et al., 2006; Hommel et al., 2006). Recent findings suggest leptin action on mesolimbic DA neurons decreases the motivational and rewarding effects of wheel running (Fernandes et al., 2015). However, hyperleptinemia during pregnancy in mice increases spontaneous activity in adult offspring, with female mice displaying greater responses to hyperleptinemia compared to male mice (Pollock et al., 2015). Additionally, pups from diabetic and obese mothers display characteristics of peripheral and central leptin resistance (Chen et al., 2008; Steculorum and Bouret, 2011). From the above observations, we hypothesized maternal overnutrition before and during pregnancy would alter physical activity levels of offspring, potentially through alterations in mesolimbic dopaminergic and leptin action.

Therefore, in the offspring of female rats fed a Western diet (WD) high in fat and sucrose before and during pregnancy we examined the extent to which maternal WD reprograms the "activity-stat" to alter voluntary physical activity levels, and influences dopamine and leptin signaling in mesolimbic brain nuclei.

MATERIALS AND METHODS

Animals and experimental design

Virgin female Wistar rats (8 weeks old, n = 8 per group) were subjected to a standard chow (SD, Purina Formulab Diet 5008) or Western (WD, Teklad TD.88137, 42% kcal from fat, 27% kcal from sucrose) diet for 5 weeks before and throughout pregnancy. For mating, a male rat (14-16 weeks old, n = 16) eating a SD was placed in the cage for up to four days. Pregnancy was confirmed by copulatory plug formation. Female rats continued the same diet intervention until term, at which time all rats received a SD. All offspring consumed a SD for the study duration. Following birth, litter sizes were culled to 10 pups (5 males, 5 females) when possible. Body weight was recorded weekly from 3 to 18 weeks of age. Male and female offspring were randomly selected from each litter (n = 5-7) and euthanized by CO_2 asphyxiation at 6 or 18 weeks of age between 1200-1500 h, prior to body fat assessment and tissue collection.

Additionally, 14 week-old SD and WD F_1 offspring (n = 5 per diet and sex) were bred to generate F_2 ND and WD offspring. As with F_1 offspring, F_2 offspring body weight was recorded weekly, and subsets of F_2 offspring (n = 5-7 per age and sex) were euthanized at 6 or 18 weeks of age for body fat assessment and tissue collection. All F_2 offspring consumed standard chow until sacrifice.

Rats maintained a 12:12-h light/dark cycle at 21-22°C, with food and water provided *ad libitum*. The Institutional Animal Care and Use Committee of the University of Missouri approved all procedures.

Offspring voluntary wheel running

Juvenile (4-7-weeks of age) and adult (16-19-weeks of age) F_1 and F_2 offspring (n = 4-7 per age and sex) were given access to voluntary running wheels (circumference = 1.08 m). Voluntary wheel running distance was monitored daily using Sigma Sport BC 800 bicycle computers.

Offspring spontaneous activity and indirect calorimetry

In 6- and 18-week-old male and female F_1 offspring (n = 4-6), a multi-dimensional beam break and indirect calorimetry system measured total energy expenditure, spontaneous activity, and food intake over a 72-hr period (Promethion, Sable Systems Int., Las Vegas, NV). Rats acclimated to chamber environment for 24 h before data collection. Data were analyzed as 24-hr averages for each animal to calculate group means.

Submaximal exercise run to exhaustion test

Run to exhaustion tests were performed in 6- and 18-week-old F_1 SD and WD offspring (n = 3-4 per age and sex). Beginning at 5 or 17 weeks of age, rats were familiarized with the treadmill for at least 5 days prior to the experiment by exposure to a 5-minute run at a speed of 15-20 m/min at a grade of 10% on a motorized treadmill (Quinton Instruments, Seattle, WA). The testing procedures in 6- and 18-week-old rats are shown in Tables 3.1 and 3.2, respectively. Exercise testing was performed between 1200 – 1400 h. An electrical shocker and blasts of air from the rear of the treadmill were used to stimulate running. Volitional exhaustion was determined as the third time a rat

remained on the electrical shocker for at least 2 seconds and could not keep pace with the speed of the treadmill, as previously described by Koch and Britton (Koch and Britton, 2001). At the moment of volitional exhaustion, the rat was removed from the treadmill and immediately sacrificed by CO_2 asphyxiation.

Table 3.1. Run to exhaustion protocol used in 6-week-old rats.

Treadmill belt speed (mph)	Grade (%)	Time (min)
20	10	60
25	10	40
30	10	Until exhaustion

Table 3.2. Run to exhaustion protocol used in 18-week-old rats.

Treadmill belt speed (mph)	Grade (%)	Time (min)
15	10	50
20	10	20
25	10	25
30	10	Until exhaustion

Body composition

Body composition was measured in 6- and 18-week-old offspring using a Hologic QDR-1000/w dual-energy X-ray absorptiometry machine calibrated for rats. Body composition was assessed in SD and WD F_0 dams just prior to breeding using isoflurane anesthesia.

Serum leptin and insulin

During sacrifice, whole blood samples were removed via heart stick, centrifuged at 1300 xg for 10 min in serum separator tubes, and stored at -20°C. Serum from non-fasted animals were assayed in duplicate for leptin concentration using a mouse/rat leptin quantitative ELISA assay (R&D Systems, Minneapolis, MN). Insulin levels were

assessed by a clinical lab.

Brain tissue extraction

NAc, VTA, and arcuate nucleus of the hypothalamus (Arc) tissues were extracted using a 2mm-thick punch tool and brain sectioning apparatus (Braintree Scientific, Braintree, MA). Tissue plugs from 2mm-thick coronal brain slices were identified per a rat brain atlas (Paxinos and Watson, 1998).

RNA isolation, cDNA synthesis, and qRT-PCR

NAc, VTA, and Arc RNA isolation, cDNA synthesis, and qRT-PCR were performed using Trizol (Qiagen) as previously described (Ruegsegger et al., 2015). Gene-specific primers were constructed using PrimerExpress3.0 (Applied Biosystems) (Table 3.3). Fifteen nanograms of cDNA from each sample were assayed in duplicate using SYBR Green Mastermix (Applied Biosystems). mRNA expression values were quantified using the $2^{\Delta\Delta Ct}$ method, whereby ΔCT = 18S Ct – target gene Ct.

Table 3.3. qRT-PCR primer sequences.

Gene	Forward (5'- 3')	Reverse (5'- 3')
18S	GCCGCTAGAGGTGAAATTCTTG	CATTCTTGGCAAATGCTTTCG
Dat	CCCCTCTGTCCACTAGCTGATG	TCCGGGAGAAACTGGCCTAT
Drd1	TCTCCTGGGCAATACCCTTGT	GGACCTCAGGTGTCGAAACC
Drd2	GTCCTGGTACGATGACGATCTG	CCTTCCCTTCTGACCCATTG
Drd5	CAACTCAATTGGCACAGAGACAA	TTGGACAGCAGGCCCTCTT
Lepr	TGGGTTTGCGTATGGAAGTC	GGTGCTTTTGTTTGGCTGTC
Oprd1	TGGTATGCACGCTCCAGTTC	GAACACGCAGATCTTGGTCACA
Oprk1	GCAATTCGCGATCGGAGC	TCCGCGGAAAATCTGGATGG
Oprm1	CGATTCCAGAAACCACATTTCA	TGTTCGTGTAACCCAAAGCAAT
Th	TGTTGGCTGACCGCACAT	CCCAGAGATGCAAGTCCAATG

Western blotting

NAc samples were pulverized and homogenized as previously described, and fifty micrograms of protein was resolved on SDS-PAGE gels for Western blotting, as previously described (Roberts et al., 2014). Primary antibodies for Drd1 (ABN20; Millipore), Drd2 (ab85367; Abcam), and Gapdh (14C10; Cell Signaling) was diluted in TBST with 5% BSA and applied to membranes overnight at 4°C. HRP-conjugated secondary antibody (7074; Cell Signaling) was applied for 1-h at room temperature in TBST with 5% non-fat milk. Prior to exposure, ECL substrate (Pierce Biotechnology) was applied, and band densitometry was obtained using a Kodak 4000R Imager and Molecular Imagery Software (Kodak Molecular Imaging Systems). Drd1 and Drd2 levels were normalized to Gapdh within each sample.

Statistical analysis

All analytical procedures were performed using SigmaPlot 12.0 (Systat Software, Inc., Chicago, IL). A two-way analysis of variance (ANOVA) assessed changes in body weight, food intake, body fat, leptin, insulin, spontaneous activity, energy expenditure, and wheel running in male and female offspring. Student's t-test assessed between group differences in mRNA and protein levels within male or female offspring at 6- or 18-weeks-old, and differences in dam characteristics. Holm-Sidak post-hoc analyses were applied when necessary. Pearson's correlation analysis was used to compare variables of interest. Full statistics from ANOVA tests are presented in Table 3.4. Significance was set at $p < 0.05$.

RESULTS

Maternal WD causes age- and sex-specific effects on F_1 offspring body composition, body weight, and serum leptin and insulin

Characteristics of SD and WD dams are shown in Figure 3.1. Of note, WD dams had 80% greater body fat percentage immediately prior to breeding ($p < 0.001$).

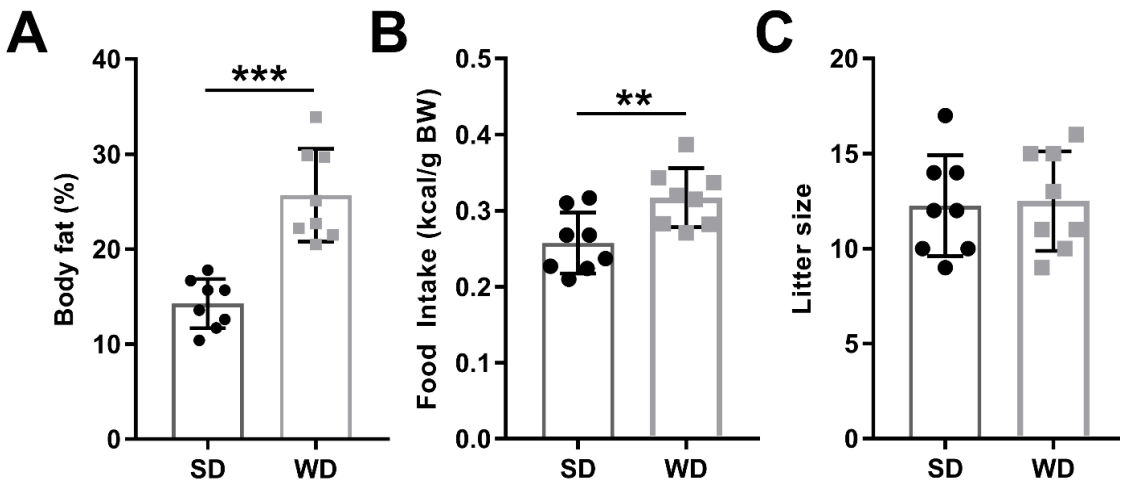

Figure 3.1. WD was obesogenic in dams. Following 5 weeks of SD or WD consumption, and just prior to breeding, body fat percentage (A) and food intake relative to body weight (B) were increased in WD compared to ND. No differences in litter size were observed between SD and WD dams. Data are mean ± standard deviation. Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Maternal WD decreased body weight in male and female F_1 offspring up to 5 and 8 weeks of age, respectively ($p < 0.05$) (Figure 3.2A, H). However, maternal WD exposure increased body weight by 18 weeks of age in male offspring ($p < 0.05$). Additionally, maternal WD increased body fat percentage, serum leptin, and serum insulin in 18-week-old male, but not female, F_1 offspring ($p < 0.01$) (Figure 3.2B-D, I-K). However, these maternal WD-induced increases in body fat, insulin, and leptin in male F_1 offspring were reduced by voluntary wheel running from 16-19 weeks of age ($p < 0.01$)

(Figure 3.3A-C). Body fat and insulin levels were greater at 18, compared to 6, weeks of age in male F_1 offspring ($p < 0.001$) while leptin was greater 18, compared to 6, weeks of age in both male and female F_1 offspring ($p < 0.01$). Metabolic chamber analysis, without wheel access, suggested that adulthood increases in body fat, leptin, and insulin levels in male WD F_1 offspring were likely the result of increased food intake ($p = 0.004$), rather than reductions in locomotor activity ($p = 0.71$) or energy expenditure ($p = 0.089$) (Figure 3.2E-G). In female F_1 offspring no differences in spontaneous activity ($p = 0.23$) or total energy expenditure ($p = 0.67$) were observed when housed without running wheels, while food intake trended to be increased in WD offspring ($p = 0.056$) (Figure 3.2L-N).

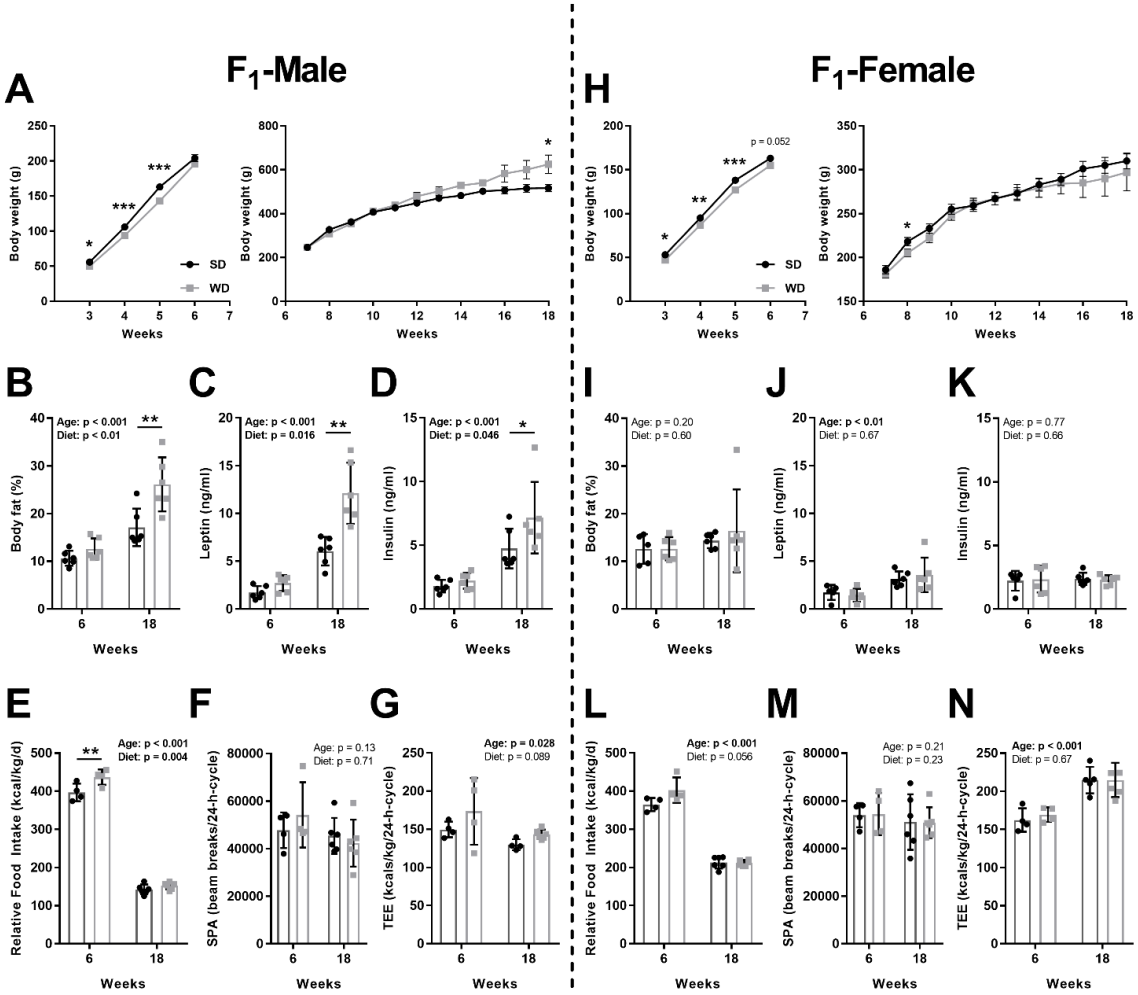

Figure 3.2. Effect of maternal WD on F_1 offspring phenotype. Maternal WD male offspring had decreased body weight up to 5 weeks of age and increased body weight at 18 weeks of age (A). Body fat (B), serum leptin (C), and serum insulin (D) were increased at 18 weeks of age in WD male offspring, despite no differences being present at 6 weeks of age. In male offspring, metabolic chamber analysis at 6 and 18 weeks of age found increased food intake relative to bodyweight in WD offspring (E), but no differences in spontaneous physical activity (SPA) (F), or total energy expenditure (TEE) (G). As observed in male offspring, Maternal WD female offspring had decreased body weight up to 7 weeks of age (H). No between diet differences in body fat (I), serum leptin (J), and serum insulin (K) were observed in 6- and 18-week-old old female WD compared to SD offspring. Metabolic chamber analysis at 6- and 18-week-old female offspring found no differences in food intake relative to bodyweight (L), SPA (M), or TEE (N) between SD and WD offspring. Data in bar graphs are mean ± standard deviation while data in line graphs are mean ± SEM. Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

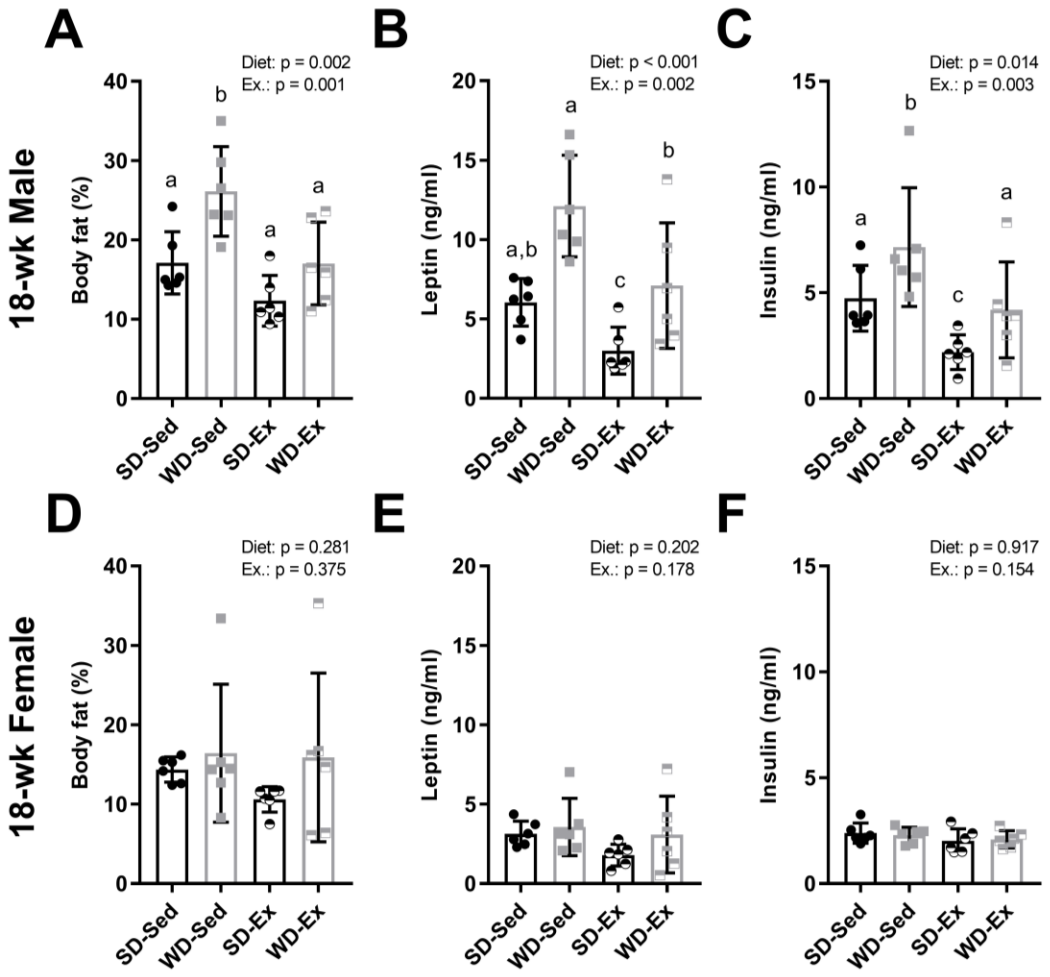

Figure 3.3. Effects of voluntary running on adiposity. Voluntary wheel running reduced body fat (A), serum leptin (B), and serum insulin (C) in adult (18 week old) male SD and WD F_1 offspring. Of note voluntary wheel running in male offspring of WD-fed dams rescues levels to to those of sedentary offspring from SD-fed dams. However, similar reductions in adult female offspring body fat (D), serum leptin (E), and serum insulin (F) were not observed following voluntary wheel running. Different letters denote statistically significant differences. Abbreviations: SD-sed: standard diet + sedentary, SD-Ex: standard diet + voluntary running wheel, WD-sed: Western diet + sedentary, WD-Ex: Western diet + voluntary running wheel. Data are mean ± standard deviation.

Maternal WD increases voluntary wheel running in juvenile female F_1 offspring

Previous studies show that developmental programming sex-specifically affects physical activity (both home cage activity and voluntary wheel running (Baker et al., 2015; Eclarinal et al., 2016; Li et al., 2013). In juvenile F_1 offspring, maternal WD did

not influence wheel running distance in male offspring ($p = 0.194$) (Figure 3.4A). Similarly, no differences in mRNA expression or Drd1 and Drd2 protein levels in the NAc were observed in 6-week-old male F_1 offspring (Figure 3.4B-D). However, in female F_1 offspring, maternal WD increased wheel running in juvenile offspring ($p = 0.003$) (Figure 3.4E), consistent with previous literature demonstrating female-specific influences of developmental programming on physical activity levels (Baker et al., 2015; Li et al., 2013). Additionally, Drd1 and Drd2 mRNA was up-regulated, while Dat mRNA was down-regulated, in 6-week-old female F_1 WD offspring ($p < 0.05$) (Figure 3.4F). Furthermore, Drd1 and Drd2 protein levels were greater in 6-week-old female WD offspring ($p < 0.05$) (Figure 3.4G-H), corroborating previous findings that increased Drd1 and Drd2 expression in the NAc may be predictive of increased wheel running behavior (Ruegsegger et al., 2015). Interestingly, VTA Lepr mRNA was down-regulated in 6-week-old female, but not male, WD F_1 offspring ($p < 0.01$) (Figure 3.4I-J), despite no differences in Arc Lepr mRNA in 6-week-old male and female offspring (Figure 3.4K).

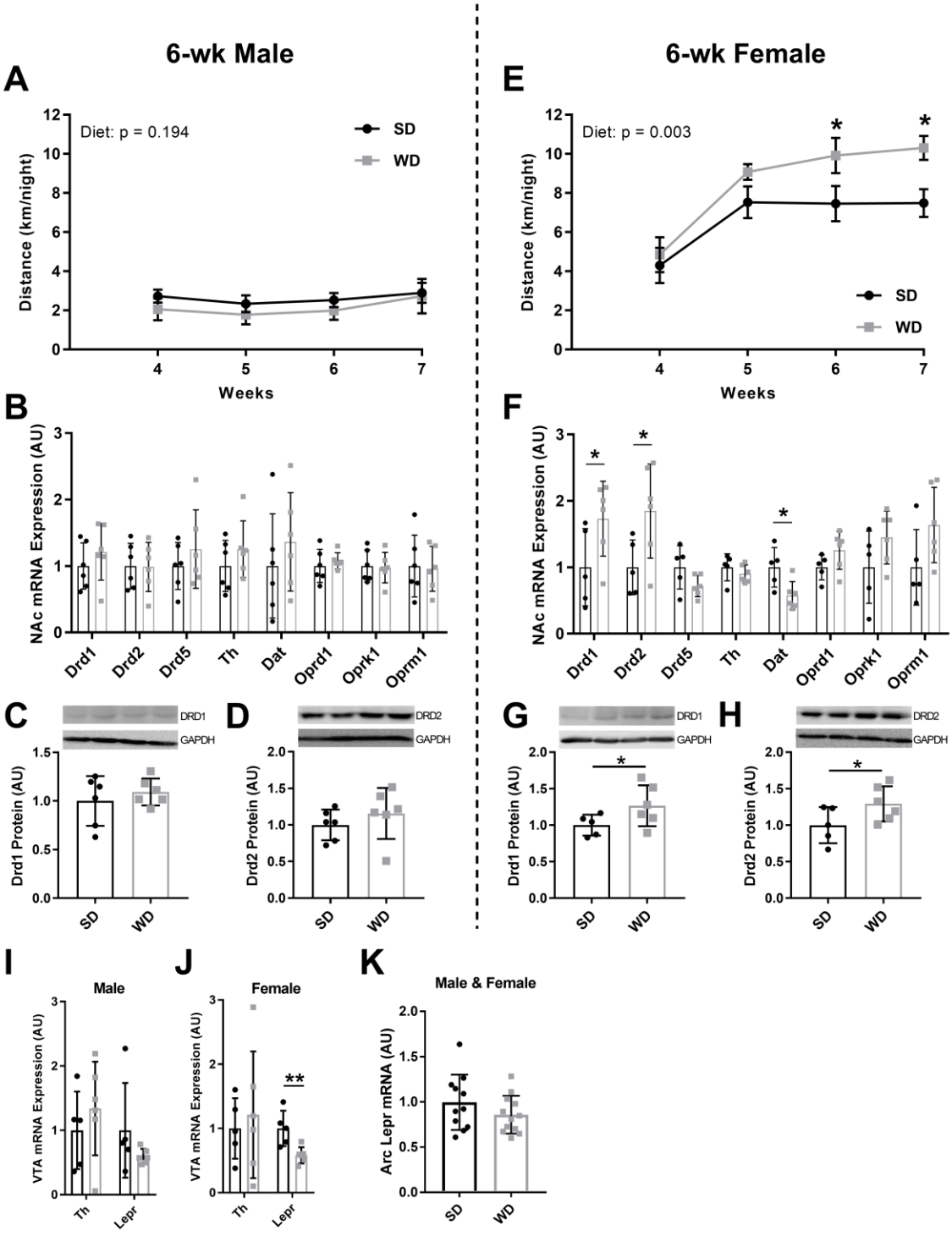

Figure 3.4. Legend is on following page.

Figure 3.4. Effects of maternal WD on voluntary wheel running in juvenile F_1 offspring. Maternal WD did not influence voluntary wheel running distance in 4-7-week-old male offspring (A). Maternal WD had no effect on NAc mRNA expression (B) or Drd1 (C) or Drd2 (D) protein levels in the NAc. Maternal WD increased voluntary wheel running distance in 4-7-week-old female offspring (E). This increase in wheel running in WD female offspring was accompanied by an increase in Drd1 and Drd2 mRNA expression and a decrease in Dat mRNA expression in the NAc (F). Similarly, Drd1 (G) and Drd2 (H) protein levels were increased in the NAc of female WD offspring compared to SD offspring. No differences in VTA mRNA expression were observed in male offspring (I), while Lepr mRNA expression in the VTA was reduced in female WD offspring (J). In analysis inclusive of both male and female offspring, no difference in Arc Lepr mRNA expression was observed between SD and WD offspring (K). Data in bar graphs are mean ± standard deviation while data in line graphs are mean ± SEM. Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Maternal WD decreases voluntary wheel running in adult female F_1 offspring

Like in juvenile F_1 offspring, maternal WD did not influence wheel running distance in adult male F_1 offspring ($p = 0.400$) (Figure 3.5A). Only NAc Dat mRNA was down-regulated in 18-week-old WD, compared to SD, male F_1 offspring ($p < 0.05$) (Figure 3.5B), and NAc Drd1 and Drd2 protein levels did not differ between SD and WD male F_1 offspring (Figure 3.5C, D). Contrary to findings in juvenile offspring, maternal WD decreased wheel running in adult female F_1 offspring ($p = 0.003$) (Figure 3.5E). Of note, differences in voluntary wheel running were independent of differences in exercise endurance during a submaximal treadmill run to exhaustion test, suggesting systems controlling exercise motivation and capacity could be dissimilar and do not contribute to differences in wheel running accompanying maternal WD (Figure 3.6). The decrease in wheel running in female WD F_1 offspring was accompanied by the down-regulation of Drd1 and Dat mRNA and up-regulation of Drd5 mRNA in the NAc of 18-week-old female WD F_1 offspring ($p < 0.05$) (Figure 3.5F). NAc Drd1 protein was decreased ($p < 0.05$) in 18-week-old female WD F_1 offspring ($p < 0.05$) without any difference in Drd2

protein ($p = 0.72$) (Figure 3.5G-H). Also opposite to 6-week-old offspring results, VTA Lepr mRNA was up-regulated in 18-week-old female, but not male, WD F_1 offspring ($p < 0.05$) (Figure 3.5I-J), while Arc Lepr mRNA in male and female 18-week-old WD F_1 offspring was down-regulated ($p < 0.001$) (Figure 3.5K). Previous findings demonstrated that interactions between leptin and the mesolimbic dopamine pathway influence wheel-running behavior (Fernandes et al., 2015). Here, serum leptin was negatively correlated with running distance in WD ($p = 0.03$), but not SD ($p = 0.79$), adult F_1-female offspring, suggesting variations in leptin sensitivity could associate with adulthood differences in wheel running following maternal ND or WD (Figure 3.5L). Similarly, analysis inclusive of both SD and WD female 18-week-old F_1 offspring demonstrated that NAc Drd1 mRNA negatively correlated with VTA Lepr mRNA ($p < 0.01$) (Figure 3.5M).

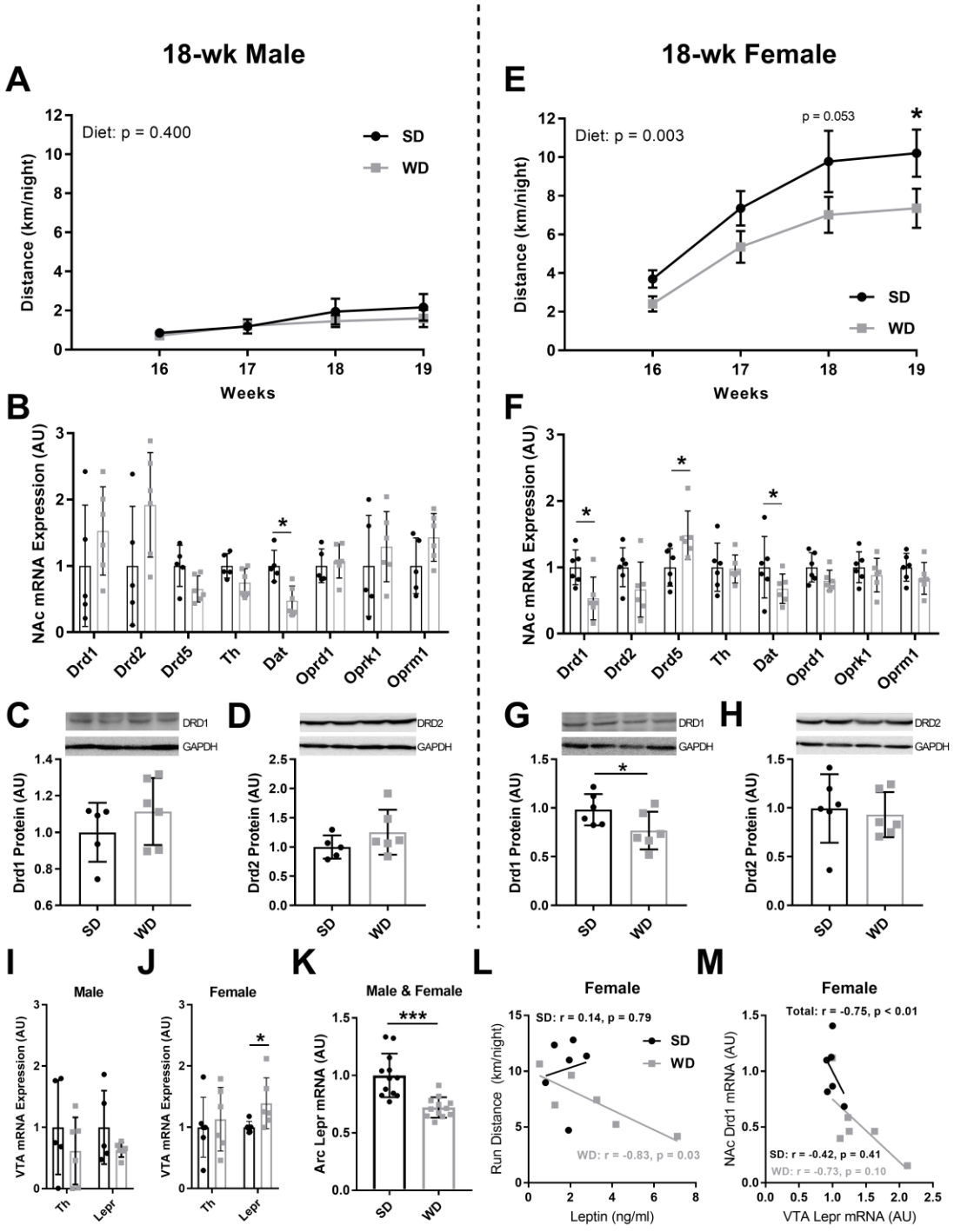

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Figure 3.5. Effects of maternal WD on voluntary wheel running in adult F_1 offspring. Maternal WD did not influence voluntary wheel running distance in 16-19-week-old male offspring (A). Dat mRNA expression in the NAc was decreased in male WD offspring (B), while Drd1 (C) and Drd2 (D) protein levels in the NAc were unchanged between SD and WD male offspring. Maternal WD decreased voluntary wheel running distance in 16-19-week-old female offspring (E). This decrease in wheel running in WD female offspring was accompanied by a decrease in Drd1 and Dat mRNA expression and an increased in Drd5 mRNA expression in the NAc (F). Drd1 (G), but not Drd2 (H), protein was decreased in the NAc of female WD offspring compared to SD offspring. No differences in VTA mRNA expression were observed in male offspring (I), while Lepr mRNA expression in the VTA was increased in female WD offspring (J). In analysis inclusive of both male and female offspring, Arc Lepr mRNA expression was decreased in WD offspring (K). Additionally, Pearson's correlation analysis showed a negative relationship between voluntary wheel running distance and serum leptin in female WD offspring (L), and a negative relationship between VTA Lepr mRNA expression and NAc Drd1 mRNA expression in SD and WD female offspring (M). Data in bar graphs are mean ± standard deviation while data in line graphs are mean ± SEM. Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

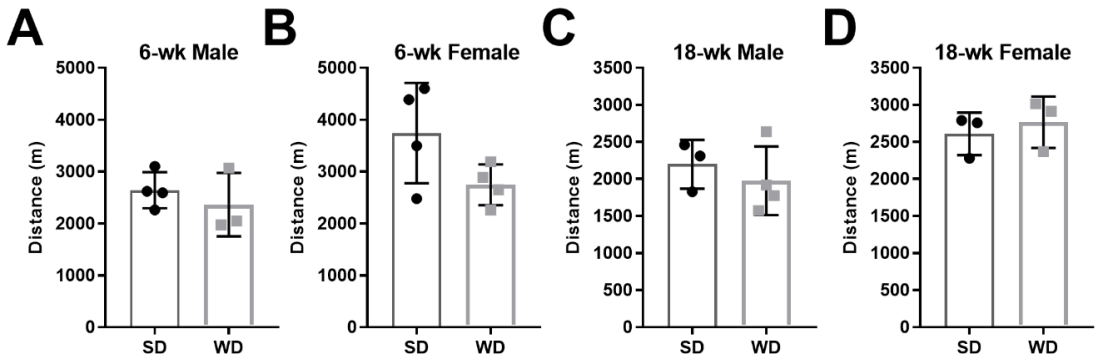

Figure 3.6. Run to exhaustion performance. Maternal WD during gestation did not influence submaximal run to exhaustion distance in 6-week-old male (A), 6-week-old female (B), 18-week-old male (C), or 18-week-old female (D) F_1 offspring. Data are mean ± standard deviation.

Maternal WD does not influence F_2 offspring voluntary wheel running

Links between maternal high-fat feeding and obesity predisposition have been observed as late as the third (F_3) generation (Dunn and Bale, 2011). However, sex-specific differences in body weight, body fat, leptin, and insulin were not present between SD and WD F_2 offspring in either male or female offspring (Figure 3.7A-D, F-I). Additionally, selective breeding experiments show transgenerational selection for voluntary running behavior (Rhodes et al., 2005; Roberts et al., 2013). However, in the present study no differences in voluntary running were present in juvenile (4-7 weeks of age) and adult (16-19 weeks of age) SD and WD F_2 male and female offspring (Figure 3.7E, J). Analysis of mRNA expression in the NAc and VTA of 6- and 18-week-old F_2 female offspring found that Dat mRNA in the NAc was decreased in WD offspring at 6 weeks of age ($p < 0.05$), while no differences in NAc Drd1, Drd2, Drd5, or VTA Lepr mRNA were observed at 6 or 18 weeks of age (Figure 3.7K, L).

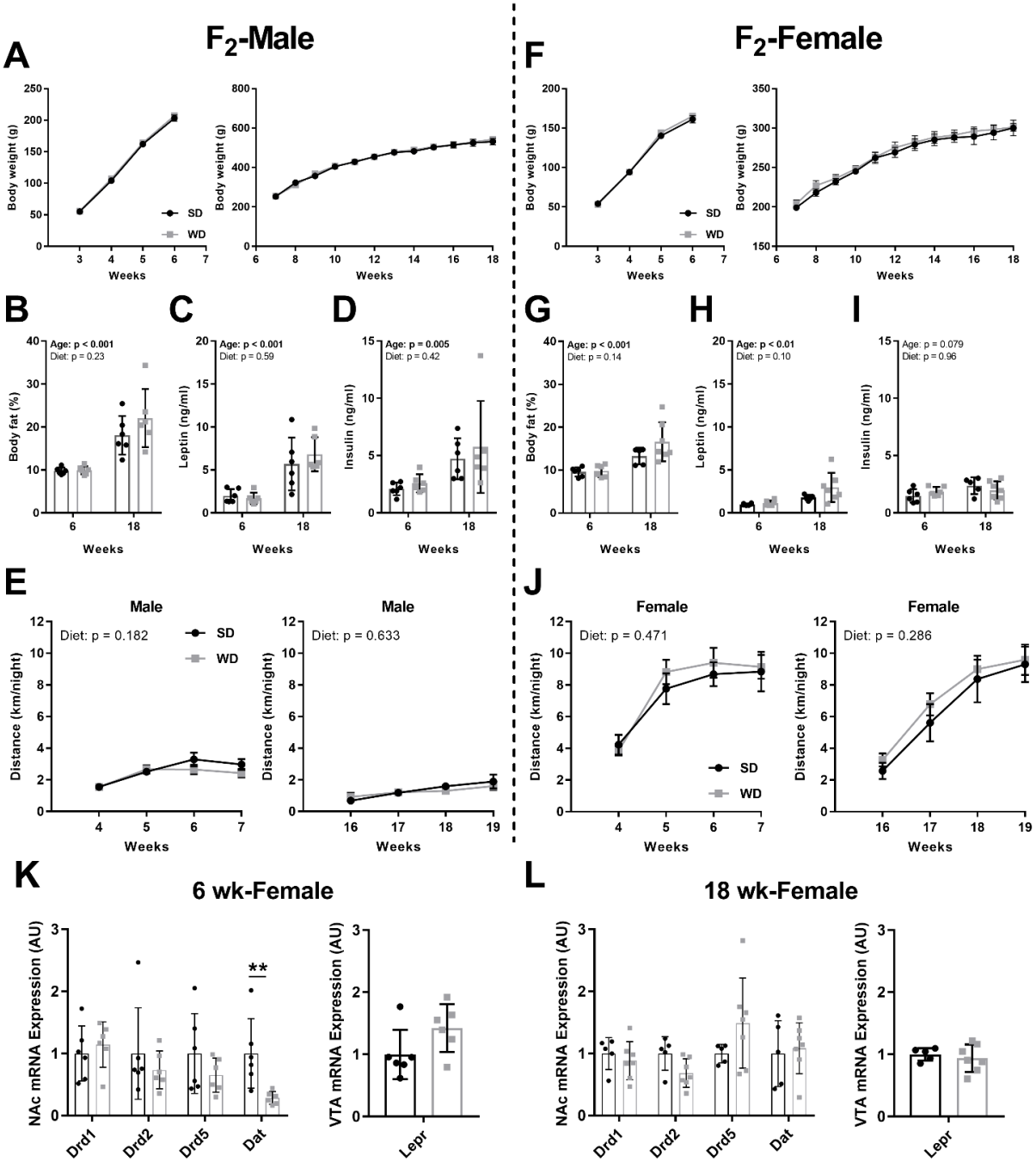

Figure 3.7. Legend is on following page.

Figure 3.7. Effect of maternal WD on F_2 offspring phenotype and voluntary wheel running. No differences in body weight (A), body fat (B), serum leptin (C), or serum insulin (D) were reported between male F_2 SD and WD offspring. Similarly, maternal WD did not influence voluntary wheel running distance in 4-7-week-old or 16-19-week-old male F_2 offspring (E). Furthermore, no differences in body weight (F), body fat (G), serum leptin (H), or serum insulin (I) were reported between female F_2 SD and WD offspring. Contrary to the F_1 generation, maternal WD did not influence voluntary wheel running distance in 4-7-week-old or 16-19-week-old female F_2 offspring (J). Analysis of mRNA expression showed that Dat mRNA was decreased in the NAc, while no differences were present in the VTA of 6-week-old female F_2 WD offspring (K). Analysis of mRNA expression in 18-week-old female F_2 offspring found no differences in NAc or VTA mRNA expression between SD and WD offspring (L). Data in bar graphs are mean ± standard deviation while data in line graphs are mean ± SEM. Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3.4. Statistical information

Measurement	Graph	Main effects/interactions	F value	P value
F_1 male body weight	2A	Age	$F_{(15.380)} = 627.14$	< 0.001
		Diet	$F_{(1,380)} = 35.94$	< 0.001
		Age x Diet	$F_{(15,380)} = 5.59$	< 0.001
F_1 male body fat	2B	Age	$F_{(1,20)} = 43.94$	< 0.001
		Diet	$F_{(1,20)} = 13.13$	0.002
		Age x Diet	$F_{(1,20)} = 5.49$	0.03
F_1 male leptin	2C	Age	$F_{(1,20)} = 47.87$	< 0.001
		Diet	$F_{(1,20)} = 6.95$	0.016
		Age x Diet	$F_{(1,20)} = 2.95$	0.10
F_1 male insulin	2D	Age	$F_{(1,20)} = 34.53$	< 0.001
		Diet	$F_{(1,20)} = 4.52$	0.046
		Age x Diet	$F_{(1,20)} = 2.21$	0.15
F_1 male relative food intake	2E	Age	$F_{(1,16)} = 1359.44$	< 0.001
		Diet	$F_{(1,16)} = 11.35$	0.004
		Age x Diet	$F_{(1,16)} = 4.34$	0.054
F_1 male spontaneous activity	2F	Age	$F_{(1,16)} = 2.57$	0.13
		Diet	$F_{(1,16)} = 0.14$	0.71
		Age x Diet	$F_{(1,16)} = 1.15$	0.30
F_1 male total energy expenditure	2G	Age	$F_{(1,14)} = 6.02$	0.028
		Diet	$F_{(1,14)} = 3.33$	0.089
		Age x Diet	$F_{(1,14)} = 0.26$	0.62
F_1 female body weight	2H	Age	$F_{(15.437)} = 452.74$	< 0.001
		Diet	$F_{(1,437)} = 10.99$	< 0.001
		Age x Diet	$F_{(15,437)} = 0.322$	0.99
F_1 female body fat	2I	Age	$F_{(1,19)} = 1.81$	0.20
		Diet	$F_{(1,19)} = 0.27$	0.60
		Age x Diet	$F_{(1,19)} = 0.24$	0.63
F_1 female leptin	2J	Age	$F_{(1,19)} = 11.89$	0.003
		Diet	$F_{(1,19)} = 0.17$	0.69
		Age x Diet	$F_{(1,19)} = 1.03$	0.32
F_1 female insulin	2K	Age	$F_{(1,19)} = 0.09$	0.77
		Diet	$F_{(1,19)} = 0.20$	0.66
		Age x Diet	$F_{(1,19)} = 0.81$	0.38
F_1 female relative food intake	2L	Age	$F_{(1,16)} = 390.78$	< 0.001
		Diet	$F_{(1,16)} = 4.27$	0.056

		Age x Diet	$F_{(1,16)} = 5.06$	0.039
F_1 female spontaneous activity	2M	Age	$F_{(1,16)} = 1.72$	0.21
		Diet	$F_{(1,16)} = 1.55$	0.23
		Age x Diet	$F_{(1,16)} = 1.54$	0.23
F_1 female total energy expenditure	2N	Age	$F_{(1,16)} = 35.17$	< 0.001
		Diet	$F_{(1,16)} = 0.19$	0.67
		Age x Diet	$F_{(1,16)} = 0.17$	0.69
F_1 18-wk Sed vs. Ex. male body fat	3A	Diet	$F_{(1,20)} = 13.32$	0.002
		Exercise	$F_{(1,20)} = 13.64$	0.001
		Diet x Exercise	$F_{(1,20)} = 1.31$	0.27
F_1 18-wk Sed vs. Ex. male leptin	3B	Diet	$F_{(1,20)} = 20.47$	< 0.001
		Exercise	$F_{(1,20)} = 12.85$	0.002
		Diet x Exercise	$F_{(1,20)} = 0.76$	0.39
F_1 18-wk Sed vs. Ex. male insulin	3C	Diet	$F_{(1,20)} = 7.29$	0.014
		Exercise	$F_{(1,20)} = 11.35$	0.003
		Diet x Exercise	$F_{(1,20)} = 0.064$	0.80
F_1 18-wk Sed vs. Ex. female body fat	3D	Diet	$F_{(1,20)} = 1.23$	0.28
		Exercise	$F_{(1,20)} = 0.82$	0.38
		Diet x Exercise	$F_{(1,20)} = 0.52$	0.48
F_1 18-wk Sed vs. Ex. female leptin	3E	Diet	$F_{(1,20)} = 1.74$	0.20
		Exercise	$F_{(1,20)} = 1.95$	0.18
		Diet x Exercise	$F_{(1,20)} = 0.44$	0.52
F_1 18-wk Sed vs. Ex. female insulin	3F	Diet	$F_{(1,20)} = 0.011$	0.92
		Exercise	$F_{(1,20)} = 2.20$	0.15
		Diet x Exercise	$F_{(1,20)} = 0.20$	0.66
F_1 6-wk male voluntary run dist.	4A	Age	$F_{(3,28)} = 0.77$	0.50
		Diet	$F_{(1,28)} = 1.78$	0.19
		Age x Diet	$F_{(3,28)} = 0.08$	0.97
F_1 6-wk female voluntary run dist.	4E	Age	$F_{(3,28)} = 12.71$	< 0.001
		Diet	$F_{(1,28)} = 10.36$	0.003
		Age x Diet	$F_{(3,28)} = 0.79$	0.51
F_1 18-wk male voluntary run dist.	5A	Age	$F_{(3,40)} = 2.94$	0.045
		Diet	$F_{(1,40)} = 0.72$	0.40
		Age x Diet	$F_{(3,40)} = 0.19$	0.90
F_1 18-wk female voluntary run dist.	5E	Age	$F_{(3,40)} = 14.17$	< 0.001
		Diet	$F_{(1,40)} = 10.23$	0.003
		Age x Diet	$F_{(3,40)} = 0.28$	0.84
F_2 male body weight	7A	Age	$F_{(1,318)} = 1419.44$	< 0.001

		Diet Age x Diet	$F_{(1,318)} = 1.79$ $F_{(1,318)} = 0.39$	0.18 0.98
F_2 male body fat	7B	Age Diet Age x Diet	$F_{(1,20)} = 37.09$ $F_{(1,20)} = 1.54$ $F_{(1,20)} = 1.31$	< 0.001 0.23 0.27
F_2 male serum leptin	7C	Age Diet Age x Diet	$F_{(1,20)} = 32.51$ $F_{(1,20)} = 0.30$ $F_{(1,20)} = 0.83$	< 0.001 0.59 0.37
F_2 male serum insulin	7D	Age Diet Age x Diet	$F_{(1,20)} = 9.95$ $F_{(1,20)} = 0.68$ $F_{(1,20)} = 0.09$	0.005 0.42 0.77
F_2 6-wk male voluntary run dist.	7E	Age Diet Age x Diet	$F_{(3,44)} = 10.39$ $F_{(1,44)} = 1.84$ $F_{(3,44)} = 1.03$	< 0.001 0.18 0.39
F_2 6-wk female voluntary run dist.	7E	Age Diet Age x Diet	$F_{(3,44)} = 16.75$ $F_{(1,44)} = 0.53$ $F_{(3,44)} = 0.29$	< 0.001 0.47 0.84
F_2 female body weight	7F	Age Diet Age x Diet	$F_{(1,176)} = 290.13$ $F_{(1,176)} = 2.54$ $F_{(1,176)} = 0.15$	< 0.001 0.11 1.00
F_2 female body fat	7G	Age Diet Age x Diet	$F_{(1,20)} = 20.96$ $F_{(1,20)} = 2.40$ $F_{(1,20)} = 1.85$	< 0.001 0.14 0.19
F_2 female serum leptin	7H	Age Diet Age x Diet	$F_{(1,20)} = 11.08$ $F_{(1,20)} = 2.94$ $F_{(1,20)} = 1..77$	0.003 0.10 0.20
F_2 female serum insulin	7I	Age Diet Age x Diet	$F_{(1,20)} = 3.42$ $F_{(1,20)} = 0.002$ $F_{(1,20)} = 2.19$	0.079 0.96 0.16
F_2 18-wk male voluntary run dist.	7J	Age Diet Age x Diet	$F_{(3,40)} = 6.11$ $F_{(1,40)} = 0.23$ $F_{(3,40)} = 0.64$	0.002 0.63 0.60
F_2 18-wk female voluntary run dist.	7J	Age Diet Age x Diet	$F_{(3,44)} = 20.93$ $F_{(1,44)} = 1.17$ $F_{(3,44)} = 0.08$	< 0.001 0.29 0.97

DISCUSSION

Here we report that prenatal WD consumption increases voluntary physical activity in juvenile, but decreases voluntary physical activity in adult, female F_1 offspring. These changes in voluntary physical activity were associated with age-dependent directional alterations in Drd1 mRNA and protein levels in the NAc as well as Lepr mRNA in the VTA. Conversely, no differences in voluntary physical activity or NAc Drd1 and VTA Lepr mRNA or protein levels were present in male F_1 offspring of WD fed dams, despite increases in body weight and adiposity by adulthood. Collectively, these findings support the hypothesis that maternal WD consumption causes age-dependent changes in mesolimbic nuclei that may influence voluntary physical activity levels in female F_1 offspring independent of changes in adiposity, as presented in Figure 3.8.

We observed that maternal WD decreased body weight in juvenile F_1 offspring. While increased body weight at weaning following maternal high-fat feeding has been observed (Vucetic et al., 2010), other studies demonstrate maternal high-fat feeding can decrease offspring body weight at weaning and post-weaning (Ferezou-Viala et al., 2007; Howie et al., 2009; Ong and Muhlhausler, 2011). Given that the WD was lower in protein than the SD in the current study (15.2% kcal vs. 26.5% kcal), the reduced postnatal growth in WD offspring could be the result of a lack of essential amino acids in dam milk production. This hypothesis is supported by findings that low protein intake during pregnancy reduces offspring body weight in early postnatal life (Bellinger et al., 2004; Bhasin et al., 2009). Additionally, maternal low protein intake during pregnancy

increases male offspring body weight by adulthood (Bhasin et al., 2009), a response very similar to that observed in F_1 male offspring in the current study.

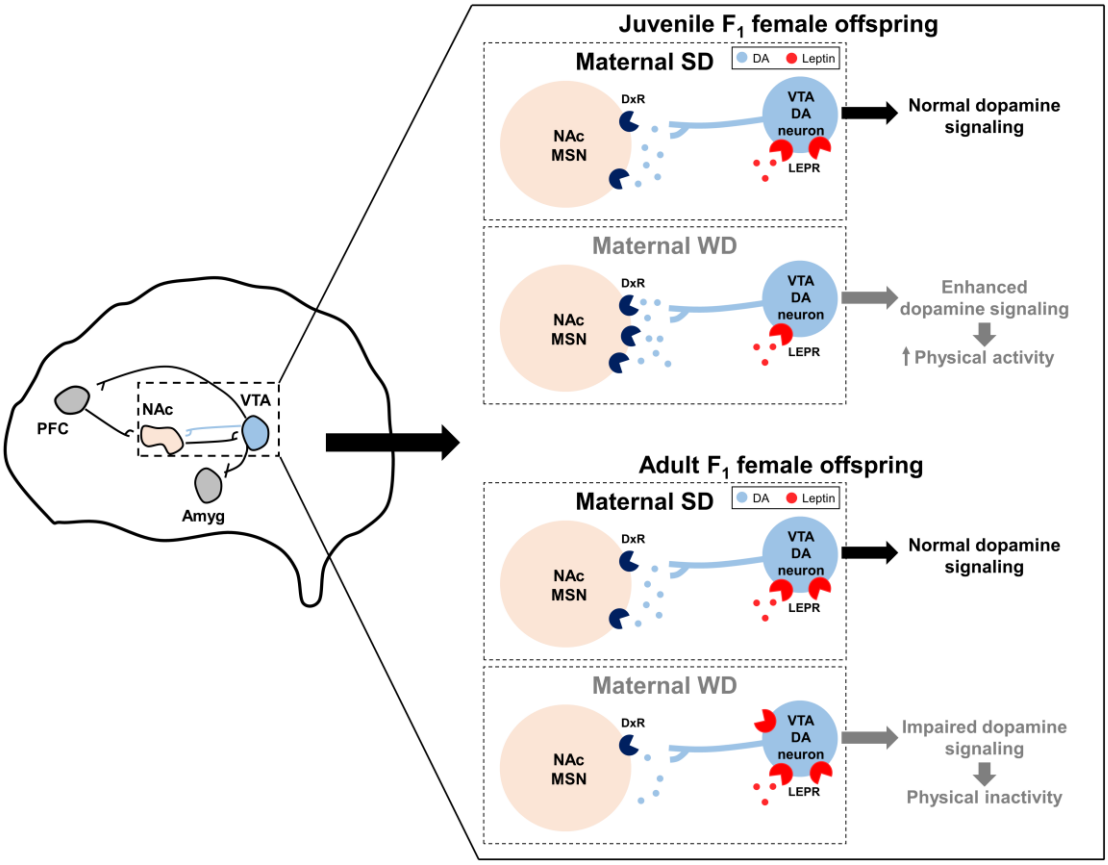

Figure 3.8. Hypothesized model by which impaired leptin and dopaminergic signaling alter physical activity in F_1 female WD offspring. Left: summary of the reward circuitry in the brain. The blue projection illustrates dopaminergic projections from the ventral tegmental area (VTA) that release dopamine (DA) onto post-synaptic neurons in the nucleus accumbens (NAc). Right: Expanded, but simplified, illustration of this dopaminergic VTA to NAc projection as it is hypothesized to relate to physical activity in F_1 SD and WD female offspring. In juvenile WD offspring, dopamine receptor (DxR), particularly Drd1, expression is decreased in the NAc Similarly, a reduction of VTA leptin receptor (LEPR) may enhance downstream DA production and release, leading to more DA in the synapse. On the contrary, in adult WD offspring , reduced NAc DxR expression and increased VTA LEPR levels could collectively lead to diminished down-stream DA function. Collectively, these impairments in dopaminergic signaling may lead to exacerbated levels of physical inactivity in adulthood.. Other abbreviations: Amyg, amygdala, PFC, prefrontal cortex.

In our model, maternal WD increased body fat, leptin, and insulin in adult male, but not female, F_1 offspring. This result supports a previous report following high-fat (60% lipids) consumption during pregnancy in Wistar rats, 9-month-old male offspring displayed mild glucose intolerance, hyperleptinemia, hyperinsulinemia, and increased visceral adiposity, while similar observation were not present in female offspring (Lecoutre et al., 2016). However, our findings are in disagreement with findings that high-fat (58%) and high-sucrose (25%) consumption during pregnancy and lactation increases body fat and decreases glucose tolerance in adult female, but not male, offspring (Dearden and Balthasar, 2014), and that high-fat diet (45%) during pregnancy and lactation increases body fat, leptin, and insulin in adult male and female offspring (Howie et al., 2009). These discrepancies highlight the potential variability that the duration of maternal high-fat/WD feeding, diet content (e.g. percentage of lipids vs carbohydrates), lipid composition (saturated vs unsaturated), and diet palatability, and thus food consumption and obesity severity, may have on fetal and/or postnatal programming. Furthermore, our finding that increased adiposity in male WD offspring is likely due to hyperphagia supports findings that maternal high-fat consumption increases food intake to a greater extent in male, than female, offspring (Desai et al., 2014). Additionally, our observation that voluntary wheel running ~1.2 km/day in adult male WD offspring reduced body fat, leptin, and insulin levels to those of sedentary SD offspring has significant clinical importance and suggests that maternal WD-induced metabolic dysfunction is malleable to voluntary exercise.

Despite its widespread importance, our understanding of how environmental factors influence voluntary physical activity remains limited. Here, wheel running was

increased in juvenile, but decreased in adult, F_1 female WD offspring, while no differences in wheel running were present in F_1 male offspring at either age assessed. Our observation that maternal WD influences physical activity in female offspring supports several prior publications in which spontaneous physical activity was reduced specifically in female offspring following prenatal and/or postnatal overnutrition (Baker et al., 2015; Khan et al., 2003; Li et al., 2013) or undernutrition (Vickers et al., 2003). Surprisingly, however, in the current study maternal WD did not alter spontaneous activity in female offspring. This discrepancy from previous reports could be attributed to the lack of body fat difference between SD and WD female offspring. Of the previously mentioned reports, two studies detected that maternal overnutrition resulted in increased adiposity which associated with reduced spontaneous activity in female offspring (Baker et al., 2015; Li et al., 2013), while adiposity was not measured in the other studies (Khan et al., 2003; Vickers et al., 2003). Collectively, these reports and our data suggest that alterations in body fat may be necessary for changes in spontaneous activity that accompany maternal overnutrition, but not for changes in voluntary physical activity, and that regulatory mechanisms controlling spontaneous and voluntary physical activity following maternal or postnatal WD are dissimilar, as has been previously speculated (Garland et al., 2017).

In the present study, increased Drd1 and Drd2 mRNA and protein in the NAc accompanied increased wheel running in juvenile, female F_1 WD offspring. This observation was reserved in adult, female WD F_1 offspring, where decreased Drd1 mRNA and protein in the NAc complemented decreased wheel running. Other (Rhodes and Garland, 2003) and our (Roberts et al., 2012) labs have shown that Drd1 antagonism

reduces wheel running. Furthermore, Drd1 mRNA is increased in rats selected for high, compared to low, wheel running (Ruegsegger et al., 2015). Therefore, we posit that one mechanism possibly explaining alterations in wheel running with maternal WD stems from increased and decreased Drd1 protein in 6- and 18-week-old female F_1 offspring, respectively. Similarly, Friend et al. (Friend et al., 2016) reported that chronic high-fat exposure in mice reduces striatal Drd2 binding and consequently reduces wheel running. Equally fascinating are results that Drd2 knockdown in mice produces an obese phenotype compared to wild-type littermates that is primarily due to decreased physical activity (Beeler et al., 2016). Future studies should determine the potential contributions of individual dopamine receptors to altered wheel running accompanying maternal WD. Unexpectedly, Dat mRNA was decreased in juvenile female and adult male and female WD F_1 offspring. However, given the consistent, and non-sex-specific, down-regulation of Dat, we speculate dopamine receptor function, rather than reuptake, could more likely influence altered running in female WD F_1 offspring. Additionally, our observations are in line with previous findings that maternal high-fat and junk food consumption affect dopamine-related mRNA expression in young (6-weeks-old) and adult (18-24-weeks-old) offspring; however, these results were observed in both male and female offspring (Ong and Muhlhausler, 2011; Vucetic et al., 2012; Vucetic et al., 2010).

Leptin receptors colocalize with dopamine neurons in the VTA, where leptin in turn inhibits dopamine activity (Fulton et al., 2006; Hommel et al., 2006). Fernandes et al. (Fernandes et al., 2015) demonstrated that intra-VTA injection leptin suppresses the rewarding effects of wheel running in mice via STAT3 activation in dopamine neurons. Intriguingly, maternal hyperleptinemia in mice increases spontaneous activity in adult

female, but not male, offspring, (Pollock et al., 2015). These observations support the next notions. Reductions in VTA Lepr mRNA in juvenile female WD F_1 offspring might enhance dopamine activity increasing voluntary wheel running, while the elevation of Lepr in adult female WD F_1 offspring could reduce dopamine activity, and in turn reduce voluntary wheel running. In contrast, similar observations in the Arc were absent, suggesting that Lepr expression in the VTA rather than hypothalamus may more strongly predict voluntary physical activity. The finding that VTA Lepr mRNA negatively correlates with NAc Drd1 mRNA in female F_1 offspring also suggests that common regulatory factors could influence leptin and dopamine function. Our observation that serum leptin levels inversely correlate with running distance in adult, female WD offspring supports findings that leptin levels inversely associate with human marathon run-time, after adjusting for BMI (Bobbert et al., 2012), further demonstrating leptin as a potential regulator of voluntary physical activity in female WD F_1 offspring. Future experiments performing intra-VTA leptin or leptin antagonist injection in SD and WD offspring will certainly further our understanding of how genetic predisposition for altered mesolimbic leptin signaling influences voluntary physical activity.

Interestingly, no differences in adiposity, leptin, insulin, or voluntary physical activity were observed in F_2 offspring. Dunn et al. (Dunn et al., 2011) proposed that changes in health outcomes in the first generation are the result of acute somatic tissue reprograming and that if germline cells are not directly impacted by prenatal overnutrition the phenotype will terminate after the first generation. Therefore, differences in adiposity and voluntary wheel running in male and female F_1 offspring, respectively, may be attributed to dysfunction in somatic tissue, rather than changes in

primordial germ cells, following direct placental exposure to maternal WD. White et al. (White et al., 2009) suggest that maternal obesity is necessary for increases in offspring body fat and insulin resistance. While we did not measure body fat percentage in F_1 breeders no differences in body fat, or serum leptin were present between SD and WD F_1 female offspring, suggesting that F_1 WD female breeders were not obese. Thus, maternal obesity may be necessary for somatic tissue reprograming in offspring. In contrary, the down-regulation of Dat mRNA in juvenile F_2 females suggest that primordial germ cell reprogramming may accompany gestational WD, although future research is needed to discern factors contributing to transgenerational differences in Dat compared to dopamine or leptin receptor expression. Furthermore, our results disagree with previous reports demonstrating that maternal HFD during pregnancy increases body size (Dunn and Bale, 2011) and insulin resistance (Dunn and Bale, 2009; Huypens et al., 2016) as late as F_3 offspring. However, these discrepancies likely stem from difference in diet duration and content.

In summary, our data are the first to show that maternal WD sex-specifically and age-specifically influences the propensity for voluntary physical activity in F_1 offspring, and importantly that this propensity associates with the reprogramming of leptin and dopamine systems in mesolimbic brain nuclei. Given the increasing levels of physical inactivity (Kohl et al., 2012) and maternal obesity (Yogev and Catalano, 2009), these novel insights provide valuable insight into how prenatal nutrition affects voluntary physical activity.

ACKNOWEDGEMENTS

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CHAPTER 4: Loss of Cdk5 function in the nucleus accumbens decreases wheel running and may mediate age-related declines in voluntary physical activity

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ABSTRACT

Increases in age are often associated with reduced levels of physical activity, which, in turn, associates with the development of numerous chronic diseases. We aimed to assess molecular differences in the nucleus accumbens (NAc), a specific brain nucleus postulated to influence rewarding behavior, of wheel running and sedentary female Wistar rats at 8 and 14 wks of age. RNA-sequencing was used to interrogate transcriptomic changes between 8 wk and 14 wk-old wheel running rats, and select transcripts were later analyzed by qRT-PCR in age-matched sedentary rats. Voluntary wheel running was greatest at 8 wks and had significantly decreased by 12 wks. From 619 differentially expressed genes, bioinformatics suggested that cAMP-mediated signaling, DARPP-32 feedback, and synaptic plasticity were greater in 8 compared to 14 wk-old rats. In depth analysis of these networks showed significant (~20-30%; $p < 0.05$) decreases in Cadm4 and p39 mRNAs, as well as their proteins from 8 to 14 wks of age in running and sedentary rats. Further, Cadm4, Cdk5, and p39 mRNAs were significantly correlated with voluntary running distance. Analysis of dendritic spine density in the NAc showed that wheel access increased spine density ($p < 0.001$), while spine density was lower in 14 wk compared 8 wk-old sedentary rats ($p = 0.03$). Intriguingly, intra-NAc injection of the Cdk5 inhibitor roscovitine, dose-dependently decreased wheel running. Collectively, these experiments suggest that an age-dependent loss in synaptic function and Cdk5/p39 activity in the NAc may be partially responsible for age-related declines in voluntary running behavior.

INTRODUCTION

Physical inactivity has reached pandemic levels in the United States and rest of the developed world. Strong evidence shows that physical inactivity is associated with 35 chronic diseases, including major non-communicable diseases such as type 2 diabetes (T2D) and coronary heart disease (CHD), and premature mortality (Booth et al., 2012). Strikingly, the World Health Organization has declared physical inactivity as the fourth leading risk factor for death worldwide, responsible for ~6% of the deaths worldwide in 2008 (Lee et al., 2012; Organization, 2010).

Several studies demonstrate that physical activity levels first fall at young ages. Troiano et al. (Troiano et al., 2008) reported a ~34% decline in physical activity between 6-11 and 12-17 years of age. A similar report observed a 40% decline in time spent performing moderate-vigorous physical activity in grade school children (Trost et al., 2002). In one analysis of 3,700 U.S. youth from the age of 6- to 11-years old, the minutes of physical activity in girls and boys dropped ~67% and ~60%, respectively (Wolff-Hughes et al., 2014). Taken together, these reports show that by ~9-11 years of age, 50% of U.S. youth are not undertaking sufficient daily physical activities for health according to U.S. Physical Activity Guidelines.

Findings from rodent studies provide similar results. Jakubczak (Jakubczak, 1969) demonstrated that declines in free wheel running activity begin early in the life of rats (66 d of age). In addition, our lab has shown voluntary wheel running initially declines at ~8 weeks of age (Ruegsegger et al., 2016). Interestingly, increases in age are associated with declining levels of physical activity in *C. elegans* (Herndon et al., 2002; Kirkwood and Finch, 2002), flies (Marden et al., 2003), and dogs (Siwak et al., 2003) suggesting that the

decrease in physical activity with ageing is fundamental to many species. Thus, it is imperative to understand the neuromolecular mechanisms that control the maturation-associated decline in physical activity motivation.

Studies in animals and humans estimate the genetic component for physical inactivity to be between 20-80% (Festing, 1977; Kaprio et al., 1981; Lauderdale et al., 1997; Lerman et al., 2002; Lightfoot et al., 2004; Lightfoot et al., 2008) and analysis from twins suggests that 31% of sedentary behavior is heritable (den Hoed et al., 2013).

Substantial evidence suggests the mesolimbic dopaminergic pathway, specifically the nucleus accumbens (NAc), plays a significant role in determining voluntary running behavior (Knab et al., 2009; Knab et al., 2012; Knab and Lightfoot, 2010). Since its characterization as the neural interface between the limbic 'motivation' and motor systems (Mogenson et al., 1980), the NAc, and its associated circuitry, has been shown to mediate many motivating and rewarding behaviors. Further, a loss of dopaminergic action has been postulated to drive age-related declines in physical activity in humans (Ingram, 2000).

In the current study we sought to examine the NAc transcriptome of wheel-running rats at 8 and 14 weeks of age to identify transcripts associated with the initial age-related decline in voluntary physical activity. This 'omics' approach was used to generate testable hypotheses by which possible NAc features may be contributing to running motivation. Based on RNA-sequencing (RNA-seq) and bioinformatics analysis suggesting that NAc neuronal function decreases from 8 to 14 weeks, follow-up studies in sedentary and wheel running rats were conducted to test this hypothesis. Additionally, in light of our RNA-seq and initial follow-up experiments, the Cdk5 inhibitor roscovitine

was infused into the NAc to assess the role it may play in determining wheel running motivation.

MATERIALS AND METHODS

Experimental animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Missouri and complied with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals.* Female, Wistar rats were used in this study (Charles River, Raleigh, NC). Thus, a limitation of our study is the inability to analyze sex differences. We employed female rats in this study due to the findings that females usually run further than males (Jones et al., 1990; Pitts, 1984). Further rationale for the use of female rats is their body mass plateau, minimizing the effect of continued body growth in male rats, and our usage of female rats balances the predominance of literature utilizing male rats.

Rats were maintained on a 12:12-h light/dark cycle at 21-22°C, with food (Formulab Diet 5008, Purina) and water provided *ad libitum* throughout all experiments.

Experiment 1: analysis of voluntary running behavior

Female, Wistar rats were weaned at 21 days of age, and were provided with access to a voluntary-running wheel beginning at 5 weeks of age (circumference: 1.062m) (Tecniplast 2154, Tecniplast, Italy), and running distance and time were monitored using Sigma Sport BC 800 bicycle computers (Cherry Creek Cyclery, Foster Falls, VA). Evidence from our laboratory suggested that voluntary-wheel running peaks

at 8 weeks of age (Ruegsegger et al., 2016). Therefore, in order to assess the hypothesized neuromolecular-reshaping that may regulate the decrease in wheel-running behavior at this age, we sacrificed rats at either: a) 8 weeks of age (8 wk; n = 7) or b) 14 weeks of age (14 wk; n = 5). We selected 14 weeks of age, after we noted a significant drop in running distance at 12 weeks of age. NAc samples from these rats were submitted for RNA-seq and protein analysis. Given that rats in the 14 wk group ran for 6 weeks more than rats in the 8 wk group, it is just as likely that differences between 8 and 14 wks are due to duration-dependent effects of chronic exercise rather than age or nightly running distance. However, our objective was to associate molecular differences at the time when wheel running first declines during the life course, thus approaches to match exercise duration were not feasible. Due to financial limitations and because our primary objective was to identify mRNAs associated with changes in wheel-running behavior, we could only perform RNA-seq only on wheel running rats. Additionally, separate groups of sedentary rats were sacrificed at 8 wk (n = 8), and 14 wk (n = 8) to assess mRNA and protein levels in target molecules identified by RNA-seq independent of voluntary wheel running. Thus, a limitation of the current study is data for many comparisons were obtained from different methods, and thus not statistically compared.

At each experimental time point, rats were sacrificed between 1700-1900, which is up to two hours prior to the dark cycle, with carbon dioxide asphyxiation. This sacrificial point was chosen as a basal observational time-point in order to avoid acute running-induced differences in mRNA expression that likely exist upon the onset of the dark cycle. Similarly, an interesting characteristic of females is the presence of the 4-day estrous cycle influencing voluntary running distance, with peak running in the 4-day

running rhythm occurring at proestrus (Anantharaman-Barr and Decombaz, 1989). Therefore, to minimize the effects of cycling hormones rats were sacrificed on the night of proestrus as determined by the peak running night in the 4-day running rhythm or vaginal cytology.

Nucleus accumbens RNA isolation and cDNA library preparation for RNA-seq

At the time of sacrifice, brains were quickly removed and NAc tissue was extracted using a 2-mm punch tool and brain sectioning apparatus (Braintree Scientific, Braintree, MA). Tissue plugs from 2-mm thick coronal brain slices, which were identified as NAc per a rat brain atlas (Paxinos and Watson, 1998), were stored at -80°C until processing. An objective of the present study was to compare RNA-seq results with a previous RNA-seq experiment analyzing the NAc of rats selectively bred for high and low motivation for wheel running (Roberts et al., 2014), which analyzed both the NAc shell and core. Thus, a limitation of our RNA-seq data is the lack of explicit distinction between the NAc shell and core. During tissue processing, samples were lyzed in NucleoSpin XS lysis (Macherey-Nagel, Bethlehem, PA, USA) buffer using a high-speed shaking apparatus (Tissuelyser, LT, Quiagen) with RNase-free stainless steel beads. RNA was subsequently extracted using the NucleoSpin XS RNA extraction kit according to the manufacturer's instructions (Macherey-Nagel, Bethlehem, PA, USA). RNA was eluted in 40 μL of RNase-free water. High integrity of each sample was confirmed using BioAnalyzer 2100 automated electrophoresis system (Bio-Rad) prior to cDNA library construction.

cDNA library preparation was performed at the University of Missouri DNA Core using the manufacturer's protocol and reagents supplied in Illumina's TruSeq RNA sample preparation kit v2, and have been previously described (Roberts et al., 2014).

Illumina sequencing and statistical analysis of RNA-seq data

The University of Missouri DNA Core performed all RNA-seq procedures, which have been described in detail elsewhere (Rustemeyer et al., 2011). Briefly, following cDNA library construction, samples were loaded into flow cells where clusters of each oligo were replicated. Following this procedure, flow cells were placed in the sequencer and fluorescently labeled bases were attached to the complementary bases of each sequence. The Illumina Genome Analyzer recorded 50 base pair reads. Reads were trimmed to ensure adaptor sequence removal and tiled to a custom reference library that had been modified and updated from the publically available *rattus norvigecus* NCBI library (method described elsewhere (Roberts et al., 2014) using NextGENe v2.4.1 (SoftGenetics, State College, PA).

mRNA expression patterns were analyzed for annotated genes using reads per kilobase per million mapped reads (RPKM) values. To examine mRNA expression differences associated with the decreased wheel-running behavior, our analysis compared the transcriptomes of 8 wk and 14 wk-old wheel-running rats. Data processing and statistical analyses were performed using Microsoft Excel v.2013. We implemented the following filters based on previous publications (Heruth et al., 2012; Roberts et al., 2013; Song et al., 2012; Zhang et al., 2012); unique mRNA sequences with: counts per million reads (CPM) minus 2-standard deviations > 0, reads per kilobase per million reads

(RPKM) > 1, fold-change value of > ± 1.2-fold, and a false discovery rate (FDR) < 0.05. Additionally, mRNAs input into bioinformatics software had a Student's t-test p-value < 0.05. While a ±1.2-fold cut-off may seem liberal compared to other recent RNA-seq publications which have used a 1.5-to-2.0 fold-change cut-off (Heruth et al., 2012; Song et al., 2012; Zhang et al., 2012), we contend that subtle mRNA differences within the NAc of 8 wk- and 14 wk-old rats exist given that a) our model is a physiological, polygenic, *in vivo* model whereby rats are being observed in an unperturbed state, and b) only 67 and 3 transcripts differed between groups at ±1.5 and ±2.0-fold change thresholds, respectively. An overview of our filtering process is shown in Figure 4.1A.

Our approach in the current study was to identify differentially expressed transcripts at time points which were at and after the observed peak lifetime, nightly voluntary wheel running distance, in order to elucidate initial transcriptomic changes that may contribute to decreases in running phenotype during the early life course. Therefore, all transcripts were correlated with running distance in order to further explore whether a given gene was associated with running behavior (r > ±0.70). While correlation does not represent causation, a high correlation between the expression of a transcript and running distance could signify the following, and warrant future interrogation: a) the transcript of interest may be a contributor to running motivation; or b) the transcript of interest is altered by attenuated running volumes.

Bioinformatics analysis of RNA-seq data

Ingenuity Pathway Analysis (IPA; Ingenuity Systems Inc., Redwood, CA, USA) was used to examine gene networks and physiological system functions in the NAc that

differed between 8 wk- and 14 wk-old wheel-running rats. In addition, the Gene Ontology (GO) Consortium database (http://www.geneontology.org) was used to examine biological, cellular, and molecular functions differing between the 8-wk and 14-wk-old wheel running rats.

qRT-PCR for RNA-seq validation and follow-up analysis in sedentary rats

Gene primers were constructed using PrimerExpress 3.0 software (Applied Biosystems) and efficiency curves were produced for all primers. Primer efficiencies ranged between 90-110% for all genes. Twenty-five nanograms of cDNA from each sample were assayed in duplicate for each of the target genes shown in Table 4.1 using SYBR Green Mastermix (Applied Biosystems, Carlsbad, CA). mRNA expression values were quantified using the $2^{\Delta\Delta Ct}$ method, whereby ΔCT = 18S Ct – gene of interest Ct.

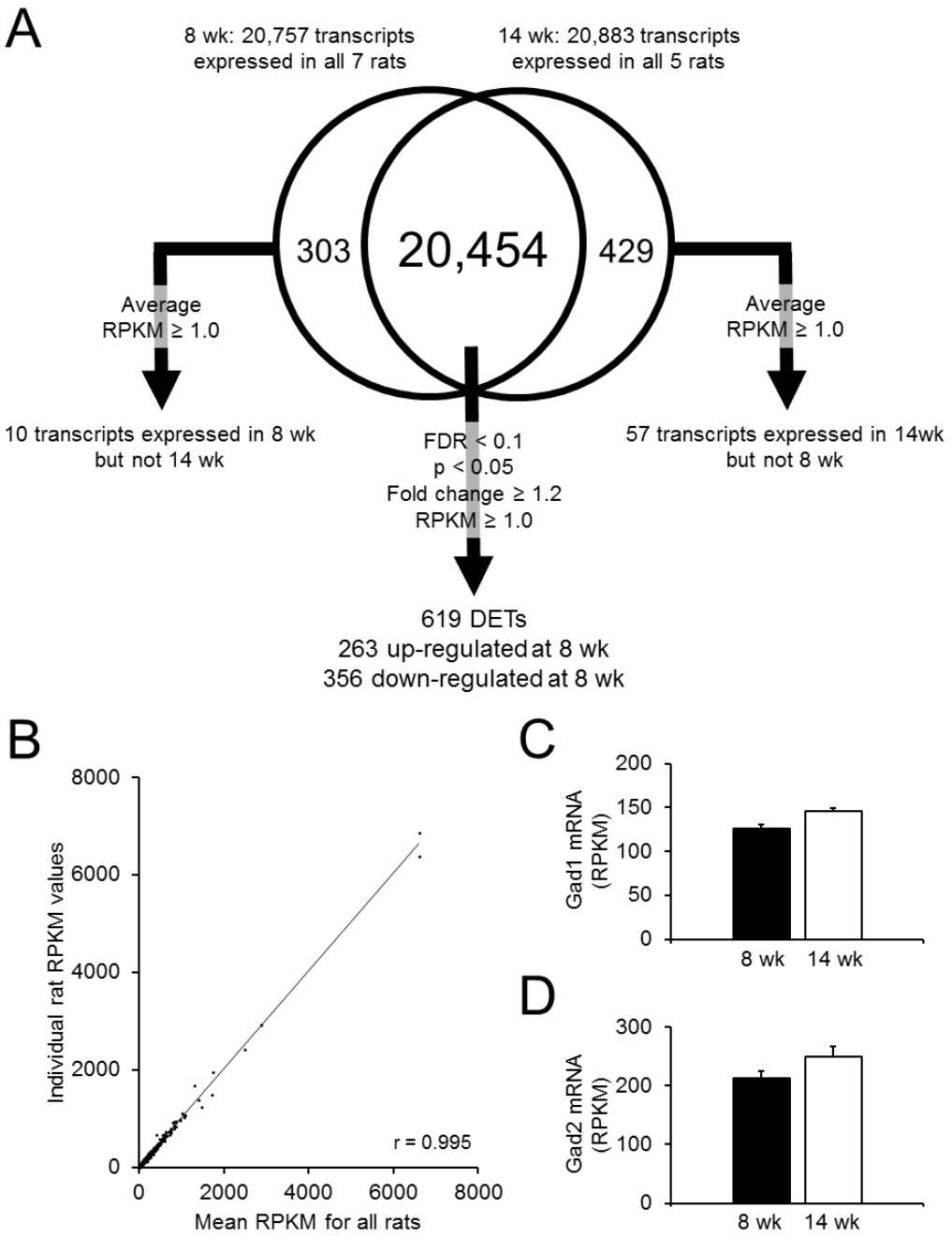

Figure 4.1. Characteristics of RNA-seq dataset and transcript filtering. (A) Overview of filtering process used to generate differentially expressed transcripts between 8 and 14 wk wheel running rats. For each group, a transcript was omitted if it was not a known transcript. (B) RNA-seq data was highly reliable as demonstrated by the high correlation (r = 0.995) of RPKM values from a single rat compared to RPKM average values from all rats. Both 8 and 14 wk groups expressed high amounts of Grd1 (*C*) and Grd2 (*D*), which is indicative of NAc MSNs. Abbreviations: False Discovery Rate (FDR); Reads per Kilobase per Million Reads (RPKM); and Differentially Expressed Transcripts (DETs).

Table 4.1. Primer sequences for gene expression analyzed by qRT-PCR

Gene	Forward (5'- 3')	Reverse (5'- 3')
18S	GCCGCTAGAGGTGAAATTCTTG	CATTCTTGGCAAATGCTTTCG
Adcy1	GAAGTCCACCGACTGCTGAA	TAGCATCTCTCCCTTGCCCT
Birc5	TGAAGGGAGGGTTGTGCAAG	ACCAACACCTACACATGGGC
Cadm4	CTGAGATCACCTGCCGTCTG	GGCTGGGTTCTGAATGACGA
Cdk5	TCTGTCACAGCCGTAACGTG	CAGCGGACTGGGATACCAAA
Cdk5r2	CCGCGGTGTCTGGATAAACT	CAGACGGAAAGGGTGAACGA
Znf238	GATGATGACCCCAGAGAGCG	CACAGGGGGCACATGAAGAT

Western blotting confirmation of RNA-seq targets up-regulated in 8 wk compared to 14 wk rats

From the same rats in which samples were submitted for RNA-seq (n = 5-7/group), and age-matched sedentary rats (n = 8/group), approximately 5-10 mg of NAc tissue was homogenized on ice in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 1x protease inhibitor cocktail] using a Tissuelyser at 25 Hz for 1 minute. The homogenate was centrifuged at 12,000xg for 10 minutes and the resultant supernatant was obtained. The BCA assay (Pierce Biotechnology, Rockford, IL) was used to determine protein concentrations. 20 µg of protein in loading buffer was loaded onto 10 or 18% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes and incubated with Ponceau S (Sigma) to verify equal loading in all lanes. Primary antibodies [rabbit polyclonal for Cadm4 (1:1,000 Abcam), p39 (1:1000, Cell Signaling), p35/25 (1:1000, Cell Signaling), and Darpp-32 (1:1000, Cell Signaling)] were diluted in TBST with 5% bovine serum albumin and applied to membranes overnight at 4°C. HRP-conjugated secondary antibody (1:1,000; Cell Signaling) was then applied to membranes for 1 hour at room temperature in TBST with 5% non-fat milk. Prior to exposure, ECL substrate (Pierce Biotechnology)

was applied for 2 minutes. A Kodak 4000R Imager and Molecular Imagery Software (Kodak Molecular Imaging Systems, New Haven, CT) were used to obtain band densitometry.

Golgi staining for MSN dendritic spine determination

For rapid Golgi staining, 8 wk- and 14 wk-old sedentary and wheel running rats (n=6/group) were sacrificed using carbon dioxide asphyxiation and brains were rapidly removed from the skull and briefly rinsed in distilled water. Golgi staining was performed using the FD rapid GolgiStain kit (FD NeuroTechnologies, Inc.) according to manufacturer's protocol. Coronal sections (200 μm) were cut with a vibrotome (Vibrotome 3000, Vibrotome Company Inc., MO). Slices were mounted on 3% gelatin-coated microscope slides (Fisher Scientific). Spine density was assessed on 10-12 dendritic segments (sampled from the entire medial NAc shell and similar rostrocaudal levels of the NAc core) per rat, sampled equally between hemispheres, resulting in the analysis of 70-72 segments per treatment condition. These dendritic segments were collected from two to three neurons per hemisphere. We restricted our study to dendritic segments of 2nd order branches, and spine density was assessed by manual labeling spines ~50 μm from the soma. Spine density was counted along the entire length of the branch and results were expressed as spine/10μm. Mean spine number per dendritic length was calculated from measurements on 2D digital images, and thus likely represent underestimates. Distinct spine morphologies were manually scored as thin, stubby, or mushroom types on dendrite segments as defined previously (Peters and Kaiserman-Abramof, 1970). For counting, images were coded and counted by a blinded individual.

Sections were visualized using an Olympus BX60 photomicroscope (Olympus, Melville, NY) at an overall magnification of 1000x and photographed with a Spot Insight digital camera (Diagnostic Instruments, Sterling Heights, MI). ImageJ Fiji software (National Institutes of Health, Bethesda, MD) was used to assess dendritic spine density.

Experiment 2: Follow-up intra-accumbens cannula injection

Based upon the results of the above mentioned experiment, an additional set of female, Wistar rats was used to determine if intra-accumbens injection of the Cdk5 inhibitor roscovitine (APExBIO, Houston, TX) influences voluntary running behavior. Rats were weaned at 21 days of age, and provided access to voluntary running wheels beginning at 10 weeks of age. After acclimating to the running wheels, brain cannulae were surgically inserted at 13-wks of age, and injections began at ~14 wks of age. This age was chosen based on preliminary data suggesting running behavior is level between 14 and 18 weeks of age (data not shown), allowing for a 'cleaner' experimental interpretation free of natural age-related declines in voluntary running. Additionally, while results from experiment 1 suggest mRNAs and proteins central to Cdk5 function decrease from 8 to 14 wks, expression levels are still robust at 14-15 wks allowing for pharmacological inhibition of Cdk5 effects on running to be determined.

Surgery and injection protocol

On the day of the surgery, animals (250-275g) were anesthetized with an intraperitoneal injection of a ketamine (87mg/kg) and xylazine (13mg/kg) mixture. The surgical procedures used have been described previously (Roberts et al., 2012;

Ruegsegger et al., 2015). Briefly, 10-mm, 23-gauge guide cannulae were bilaterally positioned 2.5mm above the NAc core using the coordinates as follows (in mm relative to Bregma): anteroposterior (AP) 1.30, mediolateral (ML) ± 1.85 mm, dorsoventral (DV) -4.63mm (Whishaw et al., 1977). We chose to target the NAc core given previous findings suggest that the core, and not shell, is associated with wheel running behavior (Werme et al., 2002). Following surgery, animals were warmed on a 32˚C heating pad for two hours and topical Neosporin was applied around the surgical area. Following recovery from surgery, rats were placed back into their home cages with running wheels and monitored for 7 days to ensure running patterns returned to pre-surgical values. Note that the running pattern of each rat was monitored prior to surgery to determine a voluntary running periodicity and to establish anticipated high running nights for drug injections.

The injection protocol is shown in Figure 2.2A. Because of the cyclic effects of the estrous cycle on running behavior, we chose to take measurements during the night of proestrus, which is the night of peak running distance (Anantharaman-Barr and Decombaz, 1989). Roughly one week after NAc core cannulation, bilateral intra-NAc infusion of 0.5 µl of vehicle (PBS/50% DMSO) was performed to acclimatize rats to the injection protocol. An additional vehicle injection was performed four days later. This injection served as a baseline for all rats. Four days after baseline injection, rats were bilaterally injected with either vehicle (n = 8), or roscovitine: 40 nmol/0.5 µl (n = 8) or 80 nmol/0.5 µl (n = 9). Drug concentrations were determined from previous studies suggesting intra-NAc roscovitine injection influences locomotor activity (Massart et al., 2015; Taylor et al., 2007). This injection scheme was maintained for 5 days. On the 'baseline' and 1st and 5th day of continuous vehicle or roscovitine injection, running

distance (km) was recorded in 30-min increments up to 120-min post-injection. Injections took place immediately prior to the onset of the dark cycle. We chose this timeframe because running patterns are more homogeneous during this period as compared to later time periods. Unpublished observations suggest that rats immediately begin voluntary wheel running upon the start of the dark cycle, compared to an intermittent and random running pattern later in the dark cycle.

To perform the injections, rats were gently hand-restrained for 90 s and 10-mm Hamilton syringes were mounted to an infusion pump (Harvard Apparatus, Holliston, MA). 12.5-mm, 30-gauge injector cannulae were connected to Hamilton syringes with PE-10 tubing that were used to deliver vehicle or roscovitine at a rate of 0.32µl/min. The injectors remained in place for 60 s following injection completion to ensure that vehicle/drug was properly infused, and upon completion of the injections, rats were returned to their home cages to monitor nightly wheel running.

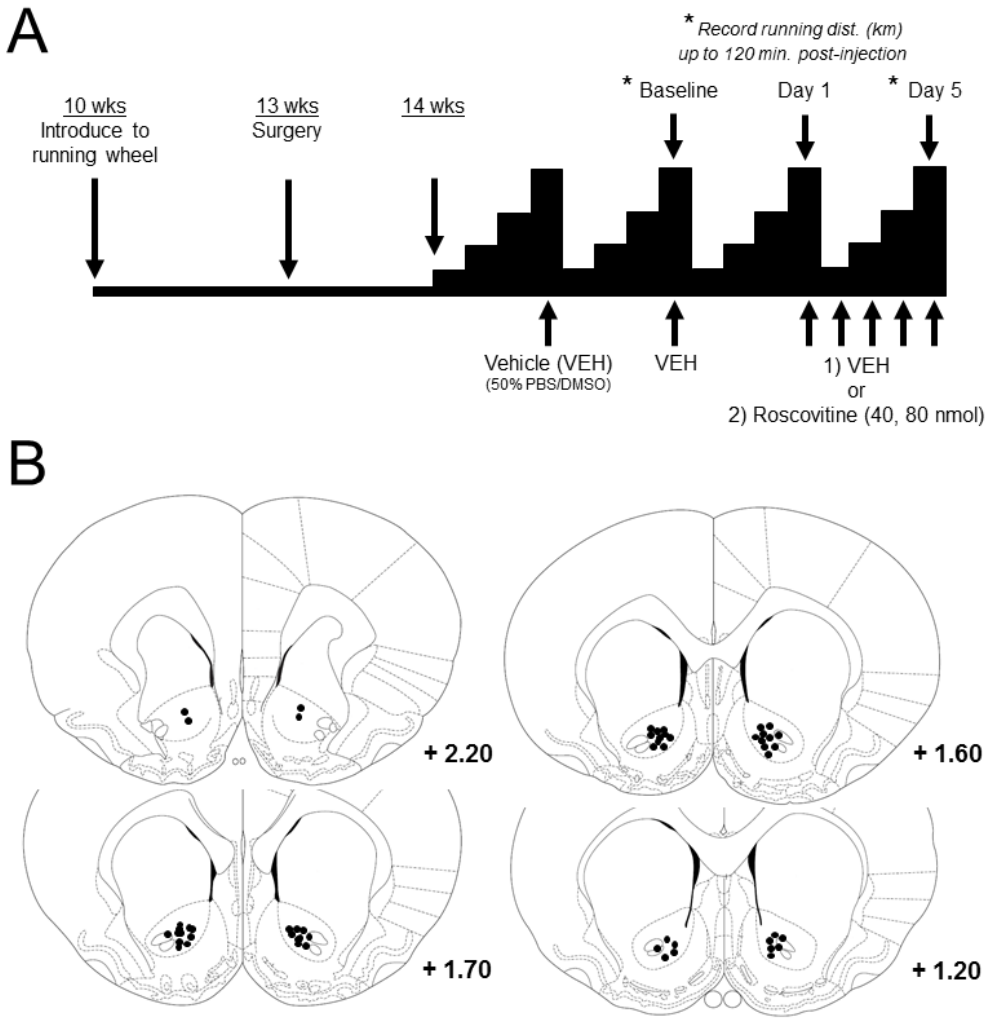

Figure 4.2. Overview of the study design for intra-NAc vehicle or roscovitine injection. (A) The schematic illustration of ascending distances of voluntary running to the peak 4th night mimics the 4-day running cycles of female rats employed in the experiment. Rats were introduced to running wheels at 10 wks of age, had cannulae surgically implanted bilaterally in the NAc core at 13 wks of age, and began the injection protocol at ~14 wks of age (wheel running was monitored for 8 days post-surgery to ensure that surgery did not disrupt running pattern). Baseline vehicle (VEH) injection was performed in all rats. Rats were then randomly administered VEH, 40 nmol roscovitine, or 80 nmol roscovitine for 5 continuous days ~10-15 min before the beginning of the dark cycle. Running distance was monitored for 120-min post-injection for the baseline injection and on the 5th night of VEH or roscovitine injection. (B) Coronal section of rat brain, as per a rat brain atlas (Paxinos and Watson, 1998), which shows the cannulae location as determined by cresyl violet staining (with position of each section given in mm relative to Bregma). Black dots represent the location of injector tips.

Verification of cannulae placement

The methods of Parker et al. (Parker et al., 2010) were used to determine cannulae placement. In brief, sections containing tracks from injectors were mounted on charged microscope slides, stained with cresyl violet, and examined using a light microscope to determine if correct cannulae placement had been made. The endpoint of each injector were mapped on a rat brain atlas (Paxinos and Watson, 1998) as presented in Figure 4.2B.

Statistical analysis

All analytical procedures were performed using SigmaPlot 12.0 (Systat Software, Inc., Chicago, IL). All values are presented as mean ± SE. Significance for all analyses was set with an α-value of 0.05. Student's t-test was used to assess between group differences in mRNA expression. Statistical analyses on band densities and dendritic spine measurements were conducted using a two-way analysis of variance (ANOVA) [Age (8wk vs. 14wk) x Treatment (Sed vs. Wheel)] with Holm-Sidak post hoc analyses when appropriate. One-way repeated measures ANOVA was used to assess age-related changes in wheel running. Two-way repeated-measures ANOVA using treatment and time as the repeated variables followed by Holm-Sidak post-hoc analyses, when necessary, was used to assess the influence of roscovitine on running distance, and percent running distance compared to baseline injection. Deviations to these statistical analyses are reported in figure legends.

RESULTS

Voluntary wheel running peaks at 8 wks of age

One-way repeated-measures ANOVA showed a significant effect of age on running distance ($F_{8,46} = 14.14$, $p < 0.001$) (Figure 4.3A). Post-hoc analysis revealed running distance was lower at 6 and 7 wks ($p < 0.001$), and at 12, 13, and 14 wks of age ($p < 0.05$), compared to 8wks. Similar results were obtained for running time ($F_{8,46} = 22.95$, $p < 0.001$) (Figure 4.3B). Repeated-measures ANOVA also showed a significant effect of running velocity ($F_{8,46} = 4.21$, $p < 0.001$), however post-hock analysis revealed differences in velocity from 8 wks only at 6 wks ($p < 0.01$) (Figure 4.3 C).

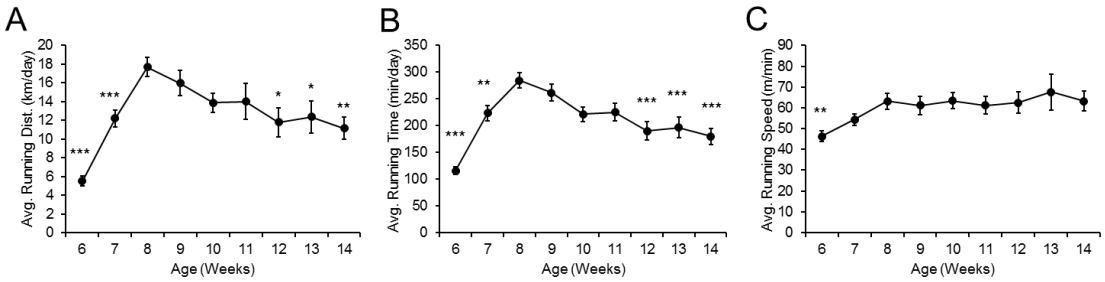

Figure 4.3. Voluntary wheel running characteristics. Average nightly running (A) distance (km), (B) time (min), (C) velocity (m/min) over the course of the study. Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Characterization of RNA-seq data from 8 wk and 14 wk wheel running rats

A summary of the total number of reads as well as the percentage of reads aligned to the reference genome is presented in Table 4.2. We observed a high correlation ($r = 0.995$) between RPKM values from an individual rat versus the mean RPKM values from all 12 rats assessed, demonstrating a high reliability of transcript detection using our RNA-seq methods (Figure 4.1B). High enrichment of glutamic acid decarboxylase 1 (Gad1) and Gad2 transcripts (RPKM, $\log_2$ values of 7-8), has been associated with NAc MSNs following laser capture microdissection (Chen et al., 2011). Our data showed high

enrichment, based on RPKM values, of Gad1 and Gad2, suggesting NAc MSNs were present in our assayed brain tissue (Figure. 4.1C, D). Additionally, differently expressed transcripts of interest were verified by qRT-PCR (Table 4.3).

Table 4.2. Summary of RNA-seq reads mapped to reference library

Group	Average total reads	Reads aligned to reference library	Total annotated transcripts expressed*
8 wk	45,040,967	84.3%	25,898
14 wk	41,524,614	83.7%	26,088

According to Chen et al. (Chen et al., 2011), 20-30 million total reads per sample in the nucleus accumbens are sufficient to provide gene expression data that correlate well with microarray and qRT-PCR data. To ensure uniform tiling across the reference genome, tiled reads from each sample were analyzed using NexGen v2.4 software. Symbols: * = RPKM > 0 in all animals within each group, as performed by Song et al. (Song et al., 2012)

Table 4.3. qRT-PCR validation of select RNA-seq transcripts

	RNA-seq		qRT-PCR	
Transcript	Fold Change (8wk/14 wk)	p-value	Fold Change (8wk/14wk)	p-value
Adcy1	1.64	0.006	1.78	0.005
Birc5	1.62	0.03	1.50	0.007
Cadm4	1.23	0.01	1.16	0.06
Cdk5r2 (p39)	1.28	0.004	1.42	0.03
Znf238	1.68	0.03	1.54	0.02

mRNA differences in 8 wk and 14 wk wheel running rats

Using the aforementioned thresholds (8 wk/14 wk ±1.2-fold, $p < 0.05$) we determined 619 NAc mRNAs were differentially expressed between 8 wk and 14 wk wheel running rats, of which 263 were up-regulated at 8 wk and 356 down-regulated at 8 wk (Figure 4.1A). Of these mRNAs, the top 10 up- and down-regulated at 8 wk/14 wk are presented in Table 4.4. Of these transcripts, Znf238 (Diotel et al., 2015), Birc5

(Survivin) (Jiang et al., 2005), Tnfaip6 (Bhattacherjee et al., 2007), and Vamp7 (Bal et al., 2013) have been associated with adult neurogenesis, brain regeneration, neural repair, survival, and development, and neurotransmission.

Transcripts meeting our filtering criteria and strongly positively or negatively correlated ($r > \pm 0.70$) with running distance during the week of sacrifice are presented in Table 4.5. Of note, the top positively correlated transcripts, Cadm2 and Cadm4, and other cell adhesions molecule family proteins, are associated with synaptic plasticity and motivation-based behaviors (Biederer et al., 2002; Robbins et al., 2010), Fa2h is essential for proper spatial learning, memory, and myelination (Potter et al., 2011), and Vamp7, which also strongly up-regulated at 8 wks, directs synaptic transmission (Bal et al., 2013).

Bioinformatics reveals up-regulation of networks pertaining to nervous system function in 8 wk vs. 14 wk running rats

IPA was used to examine pathways and networks different between 8 wk and 14 wk running rats. Of the top associated gene networks defined by IPA, the following networks were up-regulated at 8 wk vs 14 wk: 1) 'nervous system and function, organ morphology, and organismal development' (Figure 4.4) and 2) 'cellular assembly and organization, nervous system development and function, and tissue morphology'. IPA analysis of the top scoring physiological system functions also revealed up-regulation of functions pertaining to nervous system function, as well as CREB and cAMP signaling, at 8 wk vs. 14 wk (Table 4.6). Enrichment analysis of GO categories also revealed an up-regulation of functions related to nervous system function and synaptic transmission.

Table 4.4. Top ten up- and down-regulated NAc transcripts between 8 wk/14 wk

Transcript	8 wk RPKM	14 wk RPKM	Fold Change (8 wk/14 wk)	p-value	Run distance r-value
Up-regulated in 8 wk/14 wk					
Titin cap protein (Tcap)	2.16	1.23	1.75	0.04	**0.75**
Zinc finger protein 238 (Znf238)	28.09	16.67	1.68	0.03	0.62
Adenylate cyclase 1 (brain) (Adcy1)	74.38	45.21	1.65	0.001	0.68
Receptor (G protein-coupled) activity modifying protein 3 (Ramp3)	5.22	3.19	1.63	0.02	0.68
Calcium binding protein 1, transcript variant 3 (Cabp1)	34.63	21.41	1.62	0.02	**0.75**
Baculovirus IAP repeat containing 5 (Birc5)	1.80	1.12	1.62	0.03	0.31
Tumor necrosis factor, alpha-induced protein 6 (Tnfaip6)	2.71	1.68	1.61	0.03	**0.76**
Vesicle-associated membrane protein 7 (Vamp7)	6.57	4.08	1.61	0.01	**0.78**
Trophoblast glycoprotein (Tpbg)	9.10	5.74	1.58	0.01	0.57
Sterile alpha motif domain containing 9 (Samd9)	7.61	4.87	1.56	0.02	0.69
Down-regulated in 8 wk/14 wk					
Keratin 77 (Krt77)	1.21	2.65	-2.19	0.009	-0.63
BAI1-associated protein 3 (Baiap3)	7.06	14.82	-2.10	0.01	-0.49
von Willebrand factor A domain containing 5A (Vwa5a)	3.97	8.00	-2.01	0.005	-0.61
Fibronectin type III domain containing 9 (Fndc9)	1.52	3.03	-1.99	0.01	-0.59
Delta-like 1 homolog (Drosophila) (Dlk1)	5.16	10.28	-1.99	0.02	-0.56
Major histocompatibility complex, class I, A (Hla-a)	7.29	13.88	-1.90	0.001	**-0.81**
Huntingtin-associated protein 1 (Hap1)	2.93	5.48	-1.87	0.003	-0.59
Glutathione peroxidase 3 (Gpx3)	6.06	11.22	-1.85	0.01	-0.57
Neuropeptide Y receptor Y2 (Npy2r)	1.25	2.23	-1.85	0.006	-0.57
Family with sequence similarity 70, member A (Fam70a)	8.13	14.93	-1.84	0.02	-0.40

These transcripts were the top ten up- and down-regulated NAc transcripts from the 619 transcripts that were differentially expressed between animals sacrificed at 8 and 14 wks of age. Note: Run distance r-value = the Pearson correlation coefficient between distance ran in the 8wk and 14wk rats versus the RPKM value of each gene; note that a bold value signifies a strong correlation ($r > \pm 0.70$).

Table 4.5. Top NAc transcripts correlated with running distance between 8 wk and 14 wk

Transcript	Run distance r-value	p-value	Fold Change (8 wk/14 wk)
Positively correlated			
Cell adhesion molecule 2 (Cadm2)	0.87	< 0.001	1.42
Cell adhesion molecule 4 (Cadm4)	0.84	< 0.001	1.22
Fatty acid 2-hydroxylase (Fa2h)	0.83	< 0.001	1.27
UDP glycosyltransferase 8 (Ugt8)	0.82	< 0.01	1.33
Deiodinase, iodothyronine, type II (Dio2)	0.82	< 0.01	1.32
Ras viral oncogene homolog 2 (Rras2)	0.81	< 0.01	1.22
Calcium/calmodulin-dependent protein kinase II inhibitor 1 (Camk2n1)	0.79	< 0.01	1.34
Proteolipid protein 1 (Plp1)	0.78	< 0.01	1.21
Vesicle-associated membrane protein 7 (Vamp7)	0.78	< 0.01	1.61
Rab11 family interacting protein 2 (class I) (Ran11fip2)	0.77	< 0.01	1.26
Negatively correlated			
Threonine synthase-like 2 (Thnsl2)	-0.90	< 0.001	-1.24
Erythrocyte membrane protein band 4.1 like 4b (Epb41l4b)	-0.87	< 0.001	-1.31
Major histocompatibility complex, class I, H (pseudogene) (Hla-h)	-0.84	< 0.001	-1.31
dCMP deaminase (Dctd)	-0.83	< 0.01	-1.27
Kelch domain containing 8b (Klhdc8b)	-0.82	< 0.01	-1.26
Major histocompatibility complex, class I, A (Hla-a)	-0.81	< 0.01	-1.90
Monooxygenase, DBH-like 1 (Moxd1)	-0.80	< 0.01	-1.24
Filamin C, gamma (Flnc)	-0.80	< 0.01	-1.28
Major histocompatibility complex, class I, C (Hla-c)	-0.79	< 0.01	-1.78
Pygopus homolog 1 (Drosophila) (Pygo1)	-0.79	< 0.01	-1.21

These transcripts were the top ten positively and negatively correlated NAc transcripts from the 619 transcripts that were differentially expressed between animals sacrificed at 8 and 14 wks of age. Run distance r-value and p-value determined Pearson correlation coefficient between distance ran in the 8 wk and 14 wk rats versus the RPKM value of each gene.

Table 4.6. Top scoring Ingenuity Pathway Analysis biological functions and Gene Ontology terms identified as up-regulated in 8 wk vs 14 wk running rats

Term	Number of molecules	p-value	z-score / q-value
Ingenuity Pathway Analysis			
Top Physiological System Development and Functions			
Nervous System Development and Function	135	4.71E-3 – 2.14E-10	NA
Tissue Development	139	4.44E-3 – 2.14E-10	NA
Behavior	70	3.04E-3 – 9.19E-8	NA
Top Nervous System Development and Function Categories			
Neuritogenesis	43	9.64E-10	2.258
Development of neurons	53	2.14E-10	2.201
Morphogenesis of neurons	29	1.00E-6	2.023
Long-term potentiation	19	6.14E-6	2.004
Branching of neurites	16	5.63E-4	1.748
Select Activated Canonical Pathways Associated with Nervous System Development and Function			
CREB signaling in neurons	10	0.003	2.121
Dopamine-DARPP32 feedback in cAMP signaling	7	0.04	0.816
cAMP-mediated signaling	11	0.006	0.302
Gene Ontology			
Top Biological Processes			
GO:0007268 – synaptic transmission	20	6.6E-5	1.0E-3
GO:0019226 – transmission of nerve impulse	20	7.1E-6	6.0E-3
GO:0007267 – cell-cell signaling	23	4.9E-4	1.5E-1
Top Cellular Components			
GO:0044459 – plasma membrane part	62	1.9E-5	5.1E-3
GO:0045202 – synapse	18	8.9E-5	7.9E-3
GO:0030054 – cell junction	22	1.4E-4	9.6E-3
Top Molecular Functions			
GO:0000287 – magnesium ion binding	17	4.4E-3	8.9E-1
GO:0046873 – metal ion transmembrane transporter activity	13	1.0E-2	9.2-1
GO:0009055 – electron carrier activity	10	1.2E-2	8.6E-1

For IPA generated data, the z-score is used to reflect the predicted activation state of a given network or pathway. For GO generated data, the far right column displays q-values. For GO terms, only terms inclusive of at least 10 molecules per function where included in for analysis. NA: not applicable

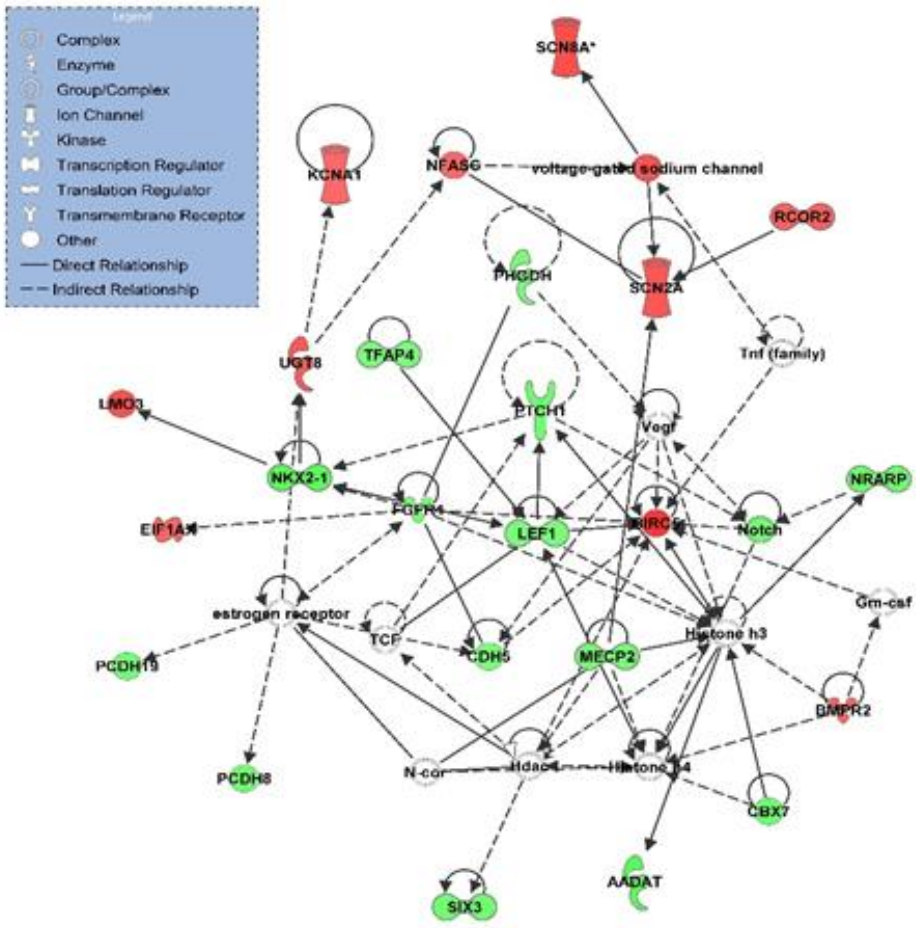

Figure 4.4. Top IPA generated network up-regulated in 8- vs. 14-week old wheel running rats. For NAc mRNAs differently expressed in 8 and 14 wk wheel running rats, the top up-regulated network at 8 wks included 'nervous system and function, organ morphology, and organismal development' (Focus molecules: 24/35, Score: 35). Nodes represent genes/molecules. Shading is proportional to fold change size (red: up-regulated, green: down-regulated). Direct and indirect relationships are denoted with solid and dashed lines, respectively. White nodes denote network members that were not altered in the network. Lines ending in an arrow or blunt end indicate known direction of molecular activation or inhibition, respectively.

Cadm4 and Cdk5-associating molecules inherently decrease from 8 wk to 14 wk and are correlated with running

Following the aforementioned IPA and GO analysis, we analyzed our RNA-seq data for mRNAs that a) associate with the IPA and GO predicted up-regulated networks, and b) have been previously associated with a low voluntary running phenotype (Roberts et al.,

2014). In doing so, we identified Cadm4 as a transcript differentially expressed between 8 wk and 14 wk running rats ($p < 0.01$) (Figure 4.5A) that 1) associates with the predicted up-regulated networks, and 2) whose expression in NAc is intrinsically greater, and correlated with running behavior, in rats selectively bred for high vs. low voluntary running (Roberts et al., 2014). Follow-up qRT-PCR between sedentary 8 wk and 14 wk rats revealed Cadm4 mRNA expression decreases from 8 wk to 14 wks of age independent of wheel running ($p = 0.026$) (Figure 4.5B). Cadm4 mRNA was also highly correlated with wheel running distance ($r = 0.84$, $p < 0.001$) (Figure 4.5C). Additionally, Western blot analysis confirmed Cadm4 protein was increased at 8 wk vs. 14 wk. independent of wheel running ($F_{1,24} = 12.10$, $p < 0.01$) (Figure 4.5D).

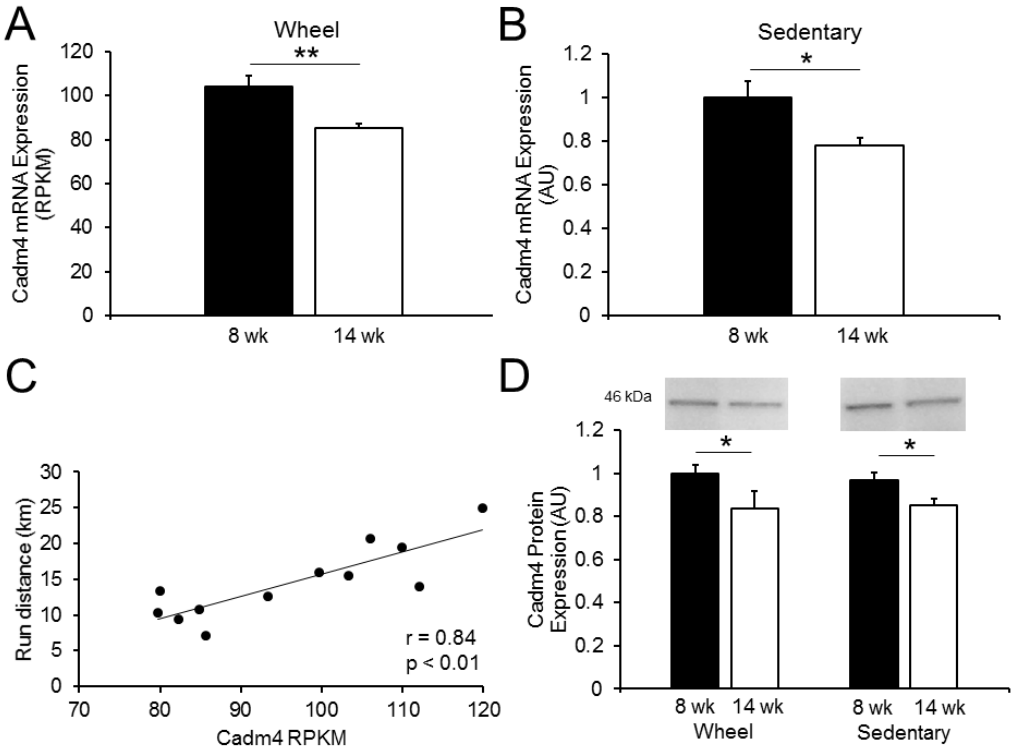

Figure 4.5. Cadm4 mRNA and protein are up-regulated at 8- vs. 14-weeks of age independent of wheel running. Cadm4 mRNA is up-regulated at 8 vs. 14 wks of age in wheel running (A) and sedentary (B) rats. Note the units in panels (A) and (B) differ due to (A) being determined by RNA-seq and (B) by qRT-PCR. Cadm4 mRNA is strongly correlated with running distance during the final week of the study (C). Similarly, Cadm4 protein decreases from 8 to 14 wks of age independent of wheel running (data normalized to 8-wk wheel running group) (D). Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Others suggest that Cdk5 is a downstream regulator of Cadm signaling pathways and promotes synaptic formation and specialization (Lai and Ip, 2009; Samuels et al., 2007). Given the role of Cdk5 in modifying cocaine-induced locomotor activity (Taylor et al., 2007), we next assessed other transcripts potentially critical for Cdk5 function. Of these transcripts, mRNA expression of the Cdk5 regulatory subunit Cdk5r2 (p39) was decreased in 14 wk vs. 8 wk wheel running ($p < 0.01$) (Figure 4.6A) and sedentary ($p < 0.01$) (Figure 4.6B) rats. p39 mRNA was also strongly correlated with running distance ($r = 0.71$, $p < 0.01$) (Figure 4.6C). Follow-up Western blot analysis revealed an age-dependent decrease in p39 protein from 8 wk to 14 wk that was independent of wheel running ($F_{1,24} = 9.54$, $p < 0.01$)

(Figure 4.6D). Assessment of a homologous Cdk5 regulatory subunit Cdk5r1 (p35) with Western blotting revealed no differences in p35 between 8 wk and 14 wk ($F_{1,24} = 0.052$, $p = 0.81$) or wheel status ($F_{1,24} = 0.25$, $p = 0.62$) (Fig. 4.6E). However, Western blotting showed an increase in p25, the cleavage product of p35, in wheel running vs. sedentary rats ($F_{1,24} = 20.98$, $p < 0.01$) suggesting increased Cdk5 activity with wheel running (Figure 4.6F).

Finally, analysis of Cdk5 mRNA expression revealed a decrease between 8 wk and 14 wk wheel running ($p < 0.05$) but not sedentary rats ($p = 0.34$) (Figure 4.6G, H). Cdk5 mRNA was also significantly correlated with wheel running distance ($r = 0.58$, $p = 0.047$) (Figure 4.6I). Together, these results suggest an age-related decline in select molecules controlling Cdk5 function may influence/modulate wheel running behavior, and that Cdk5 function may be enhanced by, and/or possibly regulate, wheel running, as previously hypothesized (Werme et al., 2002).

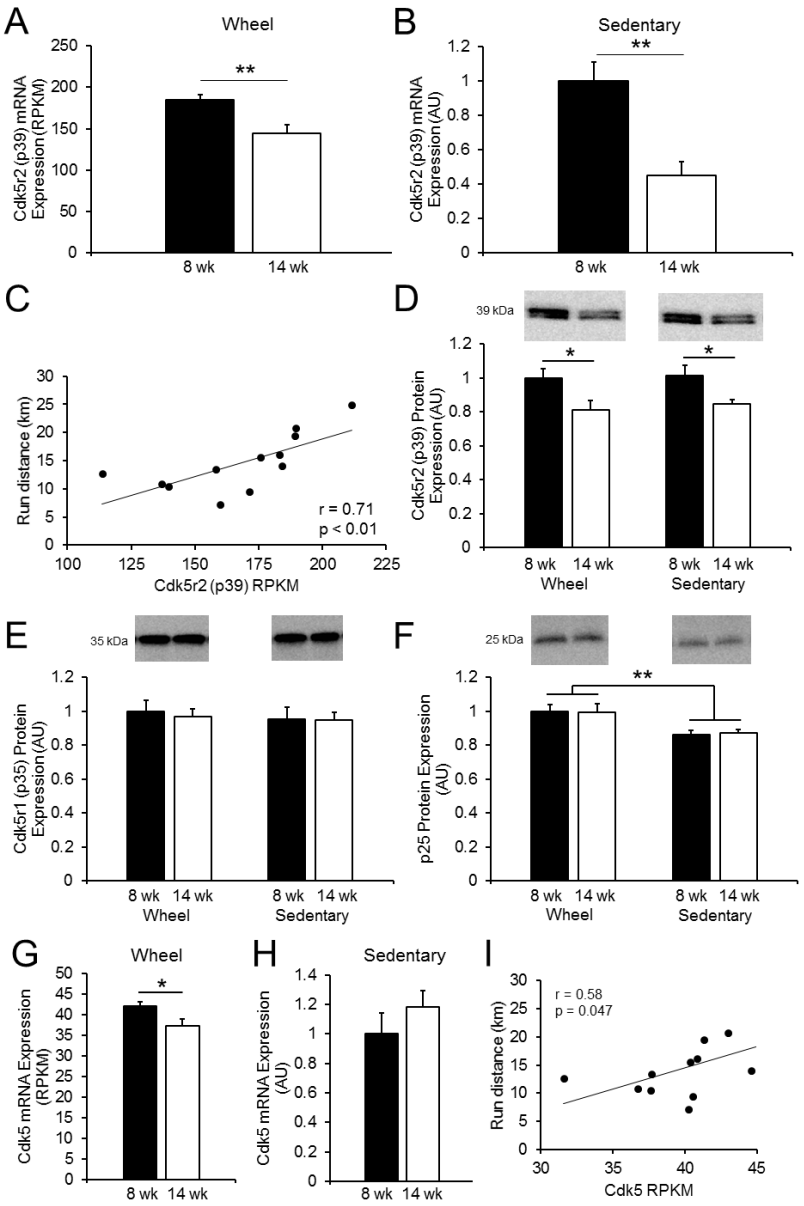

Figure 4.6. Cdk5-associated mRNA and protein differences in 8- vs. 14-week old wheel running and sedentary rats. Cdk5r2 (p39) mRNA is higher at 8 vs. 14 wks of age in wheel running (A) and sedentary (B) rats. Note the units in panels (A) and (B) differ due to (A) being determined by RNA-seq and (B) by qRT-PCR. p39 mRNA is strongly correlated with running distance during the final week of the study (C). Similarly, p39 protein decreases from 8 to 14 wks of age independent of wheel running (data normalized to 8-wk wheel running group) (D). Wheel running and age had no effect on Cdk5r1 (p35) protein level (E); however, wheel running increased protein expression of the p35 cleavage product p25 (F). Cdk5 mRNA was modestly, but significantly, greater in 8 vs. 14 wk wheel running rats (G), but was not different between 8 and 14 wk sedentary rats (H). Cdk5 mRNA was significantly correlated with running distance during the final week of the study (I). Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Neuronal maturation and dendritic density

Given the resultant IPA and GO gene networks and functions, we hypothesized that a decrease in MSN number may associate with the decrease in wheel running observed between 8 and 14 wks. Dopamine- and cAMP-regulated neuronal phosphoprotein of 32kDa (Darpp-32) protein is predominantly expressed in differentiated striatal MSNs, comprising 95% of neurons in the NAc (Arlotta et al., 2008), which we used as a marker of NAc MSN content in the current study. Western blotting showed no differences in the levels of Darpp-32 protein in 8 wk vs. 14 wk-old rats ($F_{1,24} = 1.48$, $p = 0.24$) or in wheel vs. sedentary rats ($F_{1,24} = 0.06$, $p = 0.81$) (Figure 4.7A).

Additionally, we hypothesized decreases in dendritic spine density were associated with age-related declines in wheel running. Assessment of NAc MSNs showed that the total number of dendritic spines was greater at 8 wk compared to 14 wk ($F_{1,282} = 24.28$, $p < 0.001$) and was increased by wheel running ($F_{1,282} = 51.12$, $p < 0.001$) (Figure 4.7B). We also observed a trend for an age x wheel status interaction ($F_{1,282} = 3.48$, $p = 0.063$). No differences in dendritic spine density where observed between NAc subregions (data not shown). Post-hoc analysis found a modest, but significant, decrease in dendritic spine density in sedentary rats from 8 to 14 wks, suggesting dendritic spine density in the NAc inherently decreases from 8 to 14 wks of age ($p = 0.031$). Analysis of spine type composition showed that wheel running reduced the percentage of stubby spines ($F_{1,282} = 5.94$, $p = 0.015$) while tending to increase the percentage of thin spines ($F_{1,282} = 3.84$, $p = 0.051$) (Figure 4.7C).

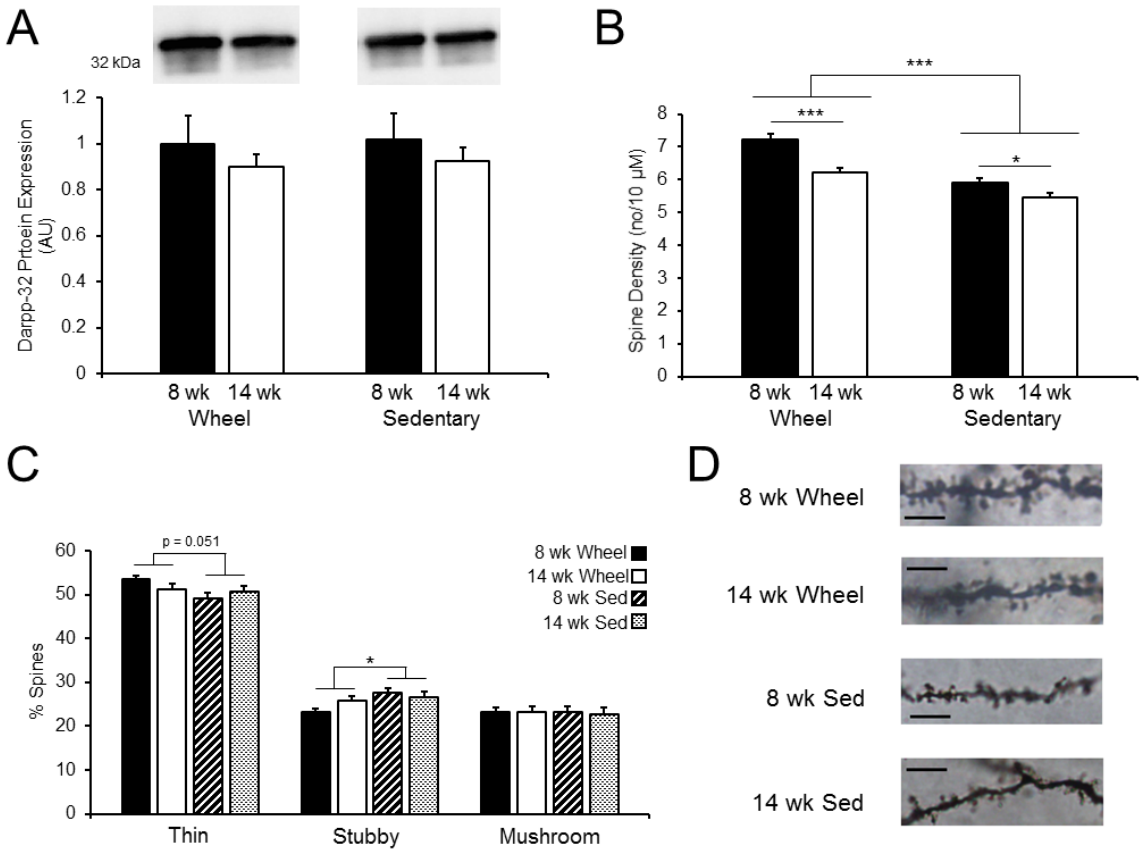

Figure 4.7. Dendritic spine density is increased in 8- vs. 14-week old wheel running and sedentary rats. (A) Darrp-32 protein was not different between any of the four experimental groups assessed. (B) Analysis of dendritic spine density showed that wheel running increased spine density, while increases in age from 8 to 14 wks was associated with a decrease in dendritic spine density. (C) Analysis of spine type showed that wheel running decreased the percentage of stubby spines and trended to increase the percentage of thin spins. (D) Representative images for the four experimental groups assessed. Scale bar = 5µM. Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Intra-NAc roscovitine injection decreases voluntary wheel running

Total running distance was significantly reduced following intra-NAc infusion of roscovitine at 40 nmol/0.5µl ($F_{2,42} = 4.93$, $p = 0.024$) and 80 nmol/0.5µl ($F_{2,48} = 19.41$, $p > 0.001$) (Fig. 8A and C). Post-hoc analysis revealed total running over 120-min test session was decreased compared to baseline after 40 and 80 nmol roscovitine on both days 1 and 5 of drug injection ($p < 0.05$). Additionally, following 80 nmol roscovitine injection, running

distance was decreased in each 30-minute interval recorded during the 120-minute trial on both days 1 and 5 ($p < 0.05$) (Fig. 4.8A).

A repeated measures ANOVA examining of the effect of roscovitine on percent change in running distance from baseline showed significant decreases in running after 40 nmol ($F_{2,41} = 5.58$, $p = 0.016$) and 80 nmol roscovitine ($F_{2,46} = 37.07$, $p < 0.001$) after the exclusion of 2 outlier points ($> \pm 2$ SD) (Fig. 4.8B). Post-hoc analysis revealed decreased percent running 0-30 and 30-60 min. post-injection following 40 and 80 nmol infusion on days 1 and 5, respectively ($p < 0.05$). Likewise, following 80 nmol roscovitine injection, percent running from baseline was decreased in each 30-minute interval recorded during the 120-minute trial on both days 1 and 5 ($p < 0.05$).

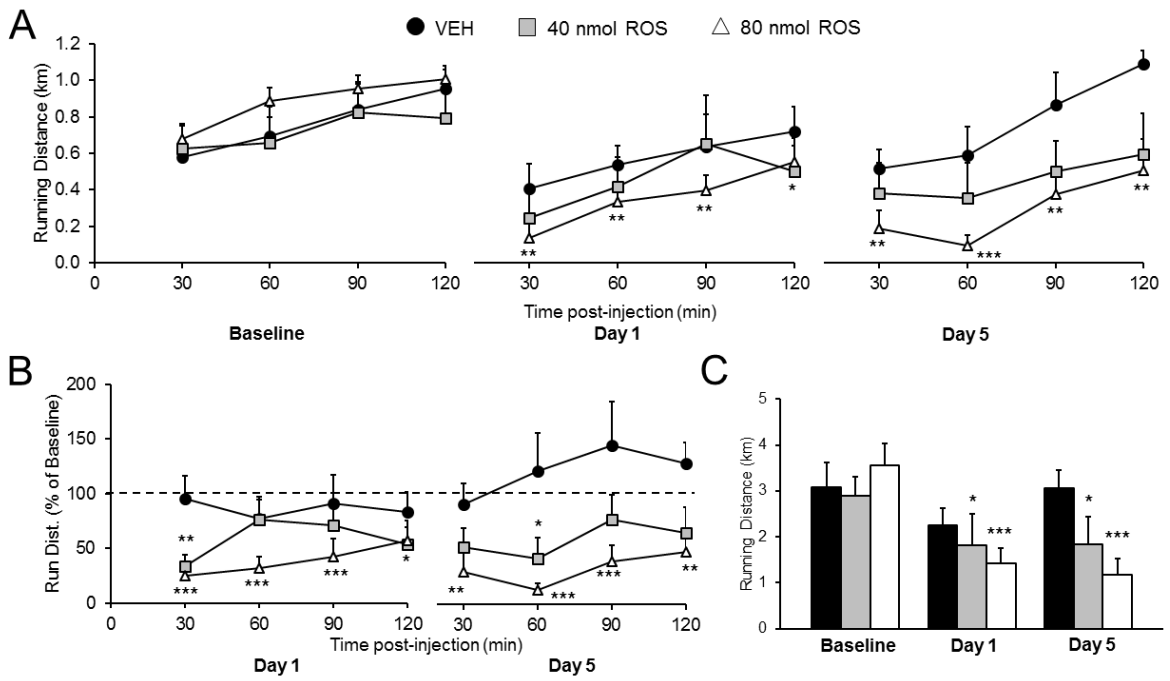

Figure 4.8. Intra-NAc roscovitine infusion decreases voluntary wheel running. (A) Running distance (km) displayed in 30-min. increments for baseline injection and on the 1st and 5th nights of vehicle (black) or 40 nmol (gray) or 80 nmol (white) roscovitine (ROS) injection. (B) Percent running distance on the 1st and 5th nights of vehicle or drug injection compared to baseline injection values. Note: two outlier points (± 2 SD) were removed from this analysis (not shown). (C) Total running distance over the 120-min. test session for the baseline injection and for the 1st and 5th night of vehicle or drug injection. All data was analyzed with repeated-measures ANOVA. Symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

In this study, our two-fold aims were: 1) to analyze molecular transducers in the NAc that may initiate and are associated with decreases in voluntary wheel running in young rats and, 2) to assess how the inhibitor of Cdk5, a molecule highlighted in our first aim, impacts wheel running behavior. We report that decreasing levels of voluntary wheel running between 8 and 14 wks are associated with declines in 1) MSN dendritic spine density, 2) gene networks critical for synaptic communication and neuron development, and 3) several transcripts central to these functions are highly correlated with wheel running distance. Additionally, declines in key mRNAs and proteins related to the above functions and

dendritic spine density were observed in analysis in age-matched sedentary rats, suggesting decrements in these functions may be the result of age, rather than from the reduction in physical activity. Finally, pharmacological inhibition of Cdk5, a molecule central to some functions described above, dose-dependently decreased wheel running. Taken together, these findings suggest, for the first time, that decreases in molecules associated with Cdk5 function in the NAc may regulate the initial decrease in wheel running behavior that begins after 8 wks of age.

Human physical inactivity is strongly associated with increased chronic disease and mortality later in life (Booth et al., 2012). Surprisingly, few studies, to our knowledge, have investigated any of the underlying biological mechanisms occurring in youth that may account for early reductions in physical activity. Furthermore, effective public health-related strategies to combat physical inactivity are relatively non-existent. Intriguingly, our observation that wheel running is greatest at 8 wks of age in female, Wistar rats matches our previous publications in male rats given wheel access at 4, rather than 6, wks of age suggesting inherent changes at 8 wks mediate running behavior in both males and females (Ruegsegger et al., 2016).

Factors associated with MSN structure are associated with age-dependent reductions in voluntary wheel running

Relationships between enhanced striatal MSN architecture and wheel running behavior have been previously established (Roberts et al., 2014). In the present study, bioinformatics using IPA and GO revealed extensive up-regulation of gene networks with functions in neuron development, dendritic spine density, and cAMP signaling in 8 wk-

compared to 14 wk-old wheel running rats; however, these results could represent alterations to reduced levels of wheel running, rather than age-induced adaptations. The extensive up-regulation of mRNAs, which have nervous system function, included ~22% of all transcripts meeting our bioinformatics-filtering criteria. Additionally, several of the top up-regulated mRNAs at 8 wks have functions specific to nervous system function, as described in the results. Likewise, one transcript only expressed at 8 wk, Arhgef7, has been implicated in the assembly of functional synapses (Zhang et al., 2003; Zhang et al., 2005), further suggesting neural plasticity and function is higher in 8-wk compared to 14-wk wheel running rats (data not shown). These findings are similar to previous findings by Roberts et al. (Roberts et al., 2014), who reported that high intrinsic levels of mRNAs indicative of increased neuronal maturation and synaptic function in the NAc are associated with high voluntarily running distances. Thus, assuming that NAc mRNA expression is an indicator of molecular transducers that are associated with voluntary wheel running behavior, we posit that these identified transcripts provide candidates that may 'predict' running behavior, and therefore begin to offer a neuromolecular basis for declining physical activity seen between 8 and 14 wks of age.

The above led us to identify molecules located at the synapse. Specifically, cell adhesion molecule 4 (Cadm4) and Cadm2 were identified as being higher at 8 vs. 14 wks, and they had high positive, strong correlations between their transcript levels and running distance. Cadm4 has been shown to promote synaptogenesis (Tanabe et al., 2013), and Cadm4 can drive the assembly of glutamatergic synapses (Biederer et al., 2002). Lower levels of Cadm4 protein have been previously associated with a low voluntary running phenotype (Roberts et al., 2014). Importantly, the overexpression of the Cadm family

member Cadm1 in mice promotes an increase in excitatory synapse number and synaptic plasticity, while loss of Cadm1 results in fewer excitatory synapses (Robbins et al., 2010). Therefore, given that synapse formation within the NAc has been attributed to addictive-like behaviors (Russo et al., 2010), we posit that the ~20% decreases in Cadm4 mRNA and protein levels could decrease the formation of specific synapses between NAc neurons and other brain regions (i.e., the ventral tegmental area, prefrontal cortex, and hippocampus as examples); an effect which may influence the reduced running behavior observed in 14 wk-old rats. Further, this decrease in Cadm4 mRNA and protein appears independent of running, and provides a potential mechanism by which age may influence the decline in wheel running between 8 and 14 wks. While a major limitation of the present study is the lack of RNA-seq on age-matched sedentary rats, the decrease in Cadm4 mRNA by qRT-PCR in the sedentary group supports a hypothesis that similar decreases in mRNAs related to synaptic function and neural plasticity decrease independent of wheel running occur between 8 and 14 wks of age; however, this must be assessed in future studies.

Previous reports suggesting that Cadm proteins regulate Cdk5 signaling pathways led us to assess Cdk5 mRNA expression in 8- and 14-wk wheel running and sedentary rats (Lai and Ip, 2009; Samuels et al., 2007). However, the Cdk5 results were not completely confirmatory. While we observed a correlation between Cdk5 mRNA and running distance in NAc, Cdk5 mRNA was only lower at 14 weeks of age, compared to 8 weeks of age, in the voluntary wheel running group. In addition, literature indicates that Cdk5 protein without its co-factors does not exhibit enzyme activity. Cdk5 co-factors Cdk5r2 (p39) (Cai et al., 1997) and Cdk5r1 (p35) (Lew et al., 1994; Tsai et al., 1994) are indispensable for Cdk5 activity. They have been shown to have similar affinity and ability to activate Cdk5 (Tang et al.,

1995). One of these, Cdk5r2 (p39) decreased at 14 wks, as compared to 8 wks, at both its mRNA and protein levels, independent of wheel running. In addition, p39 in the wheel running group was strongly positively correlated to running distance. Concerning the other Cdk5 co-factor, no differences were noted in the p35. However, wheel running increased protein expression of its cleavage product p25, suggesting that wheel running increases p35 activity. Immunohistochemical localization analysis suggests that p39 and p35 could have different functional roles in regulating Cdk5 activity, particularly in the basal ganglia (Honjyo et al., 1999). Further, Wu et al. (Wu et al., 2000) demonstrated that Cdk5 activity is ~40% less in the striatum at 6 months compared to 3 months age despite no difference in Cdk5 protein level, and this decrease was attributed to decrements in p35 and p39 expression. Together with data from the current study, these findings suggest the notion that the age-dependent declines in p39 in the striatum may drive age-related declines in Cdk5 activity.

To interim summarize the above, findings that p39 is decreased by age is particularly interesting given that Cdk5 was hypothesized to mediate increases in running behavior associated with increased expression of its transcription factor ΔFosB. Wheel running increases ΔFosB expression, likely increasing Cdk5 activity (Werme et al., 2002). Thus, it is possible that p39 decreases age-related changes in Cdk5 activity while p35/25 increases exercise-induced changes in Cdk5 activity. Finally, while Cadm and other synaptic proteins, and p39 and/or p35 likely influence Cdk5 signaling by independent mechanisms, together these findings highlight the plausibility of Cdk5 as a promising target molecule to regulate age-related declines in voluntary physical activity.

Dendritic spine density in MSNs was measured to assess whether structural differences could offer future leads to explain the aforementioned gene and network

differences. Intriguingly, we observed significant increases in spine density with wheel running. To our knowledge, this is the first report to show increases in dendritic spines in the NAc in response to wheel running. Precedence for increased spine density exists. Several reports by Stranahan et al. (Stranahan et al., 2007; Stranahan et al., 2009) have shown that wheel running increases dendritic spine density in the hippocampus. Together with findings from the present study, justification exists to assess how physical activity influences dendritic density in additional brain regions. Others have shown that increases in dendritic spine density in dopaminoceptive NAc MSNs are associated with long lasting addictive behaviors, and increases in dendritic spine density in the NAc have been observed in response to cocaine (Lee et al., 2006; Norrholm et al., 2003).

We also observed subtle changes in dendritic spine composition following wheel running, Wheel access tended to increase, and to significantly decrease the percentage of thin and stubby dendritic spines, respectively. While a non-significant trend ($p = 0.051$), the increase in thin spines following in wheel running is intriguing given their important functions in excitatory synaptic activity and dictating rapid responses to changes imposed by salient stimuli (Bourne and Harris, 2007). Similarly, LePlant et al. (LaPlant et al., 2010) demonstrated that chronic cocaine use increased the percentage of thin spines in the NAc. Hence, wheel running produces alterations in the quality of dendritic spine density in the NAc that is similar to better-characterized addictive behaviors.

We also observed significant reductions in dendritic spine density in 14 wk compared to 8 wk sedentary rats. Anderson (Anderson, 1982) observed higher dendritic densities in the medial preoptic area of the hypothalamus (MPOA), at 55 d (~8 wks of age) compared to 75 d (~11 wks of age) in rats. Like the NAc, the MPOA is highly responsive to dopamine (Hull et

al., 1995). Likewise, a recent study by Madison et al. (Madison et al., 2012) reported a ~20% reduction in dendritic spine density in the MSNs from 13 to 16 wk-old mice, further supporting the plasticity of the MSNs at relatively young ages.

Cdk5 has been implicated in many biochemical processes that influence structural and functional changes in synaptic plasticity (Cheung et al., 2006). Injection of the Cdk5 inhibitor roscovitine into the NAc reduces dendritic spine density in the NAc (Norrholm et al., 2003), further highlighting Cdk5 as a candidate target that could mediate age-dependent reductions physical activity. These differences in dendritic density in the current experiments were independent of differences in the amount of mature NAc MSNs, as assessed by Darpp-32 Western blot. However, that the ~8% drop in Darpp-32 protein between 8 and 14 weeks is comparable to the 5-10% rate of decline in the number of dopaminergic neurons in the NAc and striatum per decade in humans (Naoi and Maruyama, 1999).

Cdk5 inhibition dose-dependently decreases wheel running

To expound upon the data generated in the first experiments, we tested our notion that pharmacological inhibition of Cdk5 in the NAc core would decrease voluntary running. To our knowledge, this is the first report to observe a dose-response decrease in wheel running following infusion of the Cdk5 inhibitor roscovitine into the NAc core. The novel response to roscovitine presents important evidence that Cdk5 function in the NAc core is at least partially necessary for chronic adaptations that influence the motivation for voluntary wheel running, however voluntary wheel running is likely under polygenic control. This observation also extends our findings from the first experiment by potentially localizing a site of action for these changes to the NAc core. Together, the above add to the growing

body of literature suggesting Cdk5 activity in the NAc may be an important modulator of reward-related behaviors and highlight the neuro-chemical parallels between wheel running behavior and drug addiction.

Dopamine neurotransmission is important to multiple reward-related processes in addition to those involved in drug addiction. In the striatum, Cdk5 controls dopamine neurotransmission through the regulation of the presynaptic components of dopamine synthesis and release (Chergui et al., 2004; Kansy et al., 2004; Moy and Tsai, 2004), and Cdk5 appears to interface dopamine signaling with several downstream targets thought to mediate reward-related behavior including PKA and DARPP-32 (Bibb, 2003). Several investigations have shown that both agonism and antagonism of dopamine signaling in the NAc reduces voluntary wheel running (Knab et al., 2012; Roberts et al., 2012). Cdk5 is a natural, negative-regulator of dopamine signal transduction (Bibb et al., 1999). Rats find voluntary wheel running rewarding (Brene et al., 2007), and inhibition of Cdk5 could promote a 'substitution of reward' by replacing the reward derived through wheel running with the reward derived through potential increases in dopamine signaling. Similarly, drug intake studies blocking mesolimbic dopamine receptors have shown increased nicotine and cocaine intake (Arnold and Roberts, 1997; Corrigall and Coen, 1991), suggest that decreasing NAc dopamine may promote reward-seeking behavior and vice versa. Importantly, reductions in running following roscovitine infusion occurred on both the first and fifth night of injection. Acute roscovitine treatment evokes dopamine release (Chergui et al., 2004), suggesting that roscovitine-induced alterations in dopamine may decrease wheel running via hedonic substitution, rather than Cdk5-induced plasticity. The notion that Cdk5 influences wheel running by altering dopamine action is also similar to findings suggesting

that Cdk5 inhibition in the NAc enhances the behavioral responses to cocaine in a manner dependent of dopamine signaling in the NAc (Benavides et al., 2007; Taylor et al., 2007). Interestingly, while behavioral responses to drugs of abuse are often enhanced following roscovitine treatment, we observed a reduction in wheel running behavior, suggesting that the neuromolecular mechanisms controlling voluntary wheel running and behavioral responses to drugs of abuse are similar, but not identical.

Our finding that Cdk5 inhibition decreases wheel running also extends previous research by Werme et al. (Werme et al., 2002) showing that ΔFosB overexpression in dynorphin neurons in the striatum increases wheel running distance to suggest that Cdk5, a ΔFosB target gene, may mediate the influences of ΔFosB on wheel running. Additionally, when extending these results to age-related changes, considerable decreases between 7 and 14 weeks of age in cAMP production in NAc and striatum parallel declines in locomotor activity (Andersen, 2002), further suggesting that decreases in PKA-cAMP signaling between 8 and 14 wks may influence wheel running. Our results are also in agreement with analysis by Benavides (PhD Dissertation, UT Southwestern, 2010) who used Cdk5 conditional knockout (CKO) mice to show that after acquisition of running behavior, Cdk5 CKO mice displayed dramatically reduced steady-state levels of voluntary wheel running compared to controls. Our findings extend upon the unpublished finding to suggest that the loss of Cdk5 activity, as well as expression, mediates running behavior; however, further work is needed to identify more precisely how changes in Cdk5 activity/expression alter voluntary wheel running.

Our usage of the Cdk5 inhibitor roscovitine is limited by the possibility of nonspecific pharmacological effects and its inability to localize the effects of Cdk5 inhibition

to either presynaptic or postsynaptic compartments. An additional limitation is that inhibition of Cdk5 catalytic activity may not affect the described structural or activity-independent roles of the protein (Hawasli et al., 2007).

Conclusions

Here, we show that age-related decreases in Cadm4, p39, and MSN dendritic density are associated with age-related decreases in voluntary running, and that repeated infusion of the Cdk5 inhibitor roscovitine decreases wheel running. Our data provide guidance for future investigation into how synaptic plasticity, dopaminergic signaling, and Cdk5 function in the NAc impact voluntary wheel running. Given the epidemic levels of physical inactivity significant efforts should be taken to unravel the neuromolecular mechanisms underlying the biological motivation for voluntary physical activity.

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CHAPTER 5: Conclusions and future directions

The overall aim of this dissertation was to investigate the relationship between the mesolimbic reward pathway, primarily via molecular mechanisms in the NAc, and the motivation for physical activity. This relationship was investigated using three distinct models to assess the involvement of the mesolimbic reward pathway on wheel running. In Chapter 2, I utilized rats selectively bred from high or low voluntary wheel running distance to assess differences in endogenous opioid and DA activities, and to determine the necessity of DA to influence opioid-mediated changes in physical activity in HVR rats. In Chapter 3, I assessed the effects of maternal WD on physical activity of F_1 and F_2 offspring, mRNA expression, and protein levels of DA-related genes. Similarly, in Chapter 4, I used transcriptomic, histological, and pharmacological approaches to assess how alterations in DA action in the NAc may influence declining physical activity levels present during adolescence. Collectively, the results of these experiments suggest that alterations in dopaminergic signaling in the NAc undoubtedly associate with and/or influence voluntary physical activity, as shown in Figure 5.1. While the mechanisms by which the DA system was potentially influenced in each study likely differ (e.g. receptor expression in Chapters 2 and 3, and dendritic spine density and Cdk5 activity in Chapter 4), I find this a somewhat startling, and important, conclusion given the drastic differences among the three experimental approaches used in this dissertation. Given the potential implications by which understanding the neuromolecular mechanisms contributing to physical activity may improve the quality of life, I hope these results lead to further discoveries in this area of research.

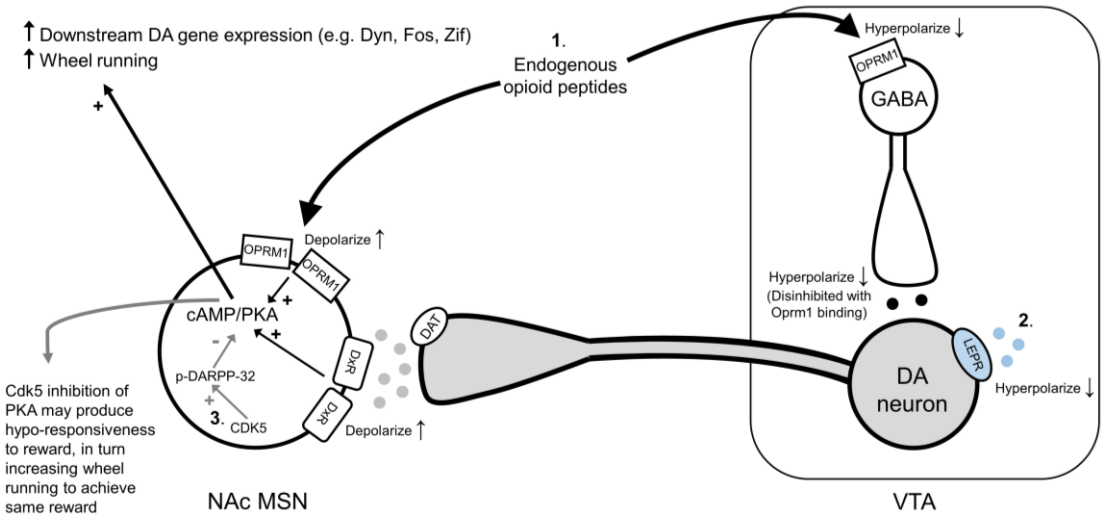

Figure 5.1. Hypothesized model summarizing how findings in this dissertation may collectively influence physical activity. 1) Endogenous opioids act on the mu opioid receptor (OPRM1) in ventral tegmental area (VTA) and nucleus accumbens (NAc). In the VTA, opioid binding to OPRM1 hyperpolarizes GABAergic neurons, in turn lowering GABA release and action on VTA dopamine (DA) neurons. This disinhibition of VTA DA neurons by OPRM1 action on GABAergic neurons increases DA synthesis in the VTA and downstream release in the NAc, where DA acts on dopamine receptors (DxR) to depolarize NAc medium spiny neurons (MSNs) and increase cAMP concentrations and PKA signaling. Activation of the PKA signaling cascade increases gene expression of immediate early genes that may enhance wheel running. Similarly, opioids bind to post-synaptic OPRM1 in NAc MSNs, producing depolarizing responses, in turn increasing cAMP/PKA signaling and downstream gene expression and wheel running. Note that opioid signaling requires interactions with dopaminergic signaling to influence wheel running, however whether these interactions are downstream of opioid action in VTA or NAc is unclear (Chapter 2). **2)** In a non-resistant state, leptin binding to leptin receptor (LEPR) hyperpolarizes VTA DA neurons, decreasing DA release on downstream NAc MSNs. The reduction in NAc DA decreases NAc cAMP/PKA signaling, potentially decreasing wheel running by lowering gene expression of immediate early genes (Chapter 3). **3)** Cyclin dependent kinase 5 (Cdk5) in the NAc phosphorylates DARPP-32, ultimately inhibiting PKA signaling. Paradoxically, data in Chapter 4 suggests that inhibiting Cdk5, and therefore increasing PKA signaling, decreases wheel running. I hypothesize, the activation of Cdk5, and subsequent inhibition of PKA signaling, may lead to hypo-responsiveness to rewarding stimuli, such as wheel running. Thus, following Cdk5 activation higher levels of wheel running may be performed to achieve the same level of reward. Given that my data suggest both activation (Chapters 2 and 3) and inhibition (Chapter 4) of NAc PKA signaling associate with increased wheel running, the mechanisms controlling wheel running appear finely controlled and highly complex.

Selective breeding highlights inherent elevations in opioid and dopamine signaling in high voluntary running rats

Physical activity is a complex, polygenic behavior in which multiple molecular adaptions likely contribute to the phenotype. Therefore, selective breeding for high and low voluntary running behavior provides a unique opportunity to study how divergences in physical activity propensity lead to the development of enriched gene signatures. In addition, knowledge of how these gene signatures associate with the polarization of extremely high and low running phenotypes provides a unique insight into heritable factors that may influence physical activity levels. In Chapter 2, I hypothesized that elevations in Oprm1 expression and function in the NAc would associate with greater reductions in wheel running following opioid receptor antagonism in HVR, compared to LVR, rats. Additionally, I hypothesized the influences of opioid receptor antagonism on wheel running were dependent on the mesolimbic dopamine system. These hypotheses were based on multiple lines of evidence showing opioids and DA are independent strong influencers of voluntary wheel running behavior (Knab and Lightfoot, 2010; Rhodes et al., 2005; Roberts et al., 2012; Ruegsegger et al., 2015; Schnur and Barela, 1984).

From the experiments in Chapter 2, I conclude 1) Oprm1 mRNA, protein, and function are increased in the NAc of HVR compared to LVR rats; 2) the opioid receptor antagonist naltrexone reduces wheel running to a greater extent in HVR than LVR; 3) systemic naltrexone injection decreases DA-related mRNAs in mesolimbic brain regions; and 4) naltrexone effects require dopaminergic nerve terminals in the NAc, and likely VTA, to influence wheel running in HVR rats. Collectively, these results suggest that inherent up-regulations in NAc opioidergic function may lead to increased physical activity levels, while

the down-regulation of opioidergic function in the NAc may promote physical inactivity, as shown in Figure 2.7. Interestingly, these results were independent of differences in peripheral pain sensitivity, an opioid-mediated function, suggesting that pain tolerance is not co-selected with the selection for voluntary running motivation in healthy rats. Additionally, given the postulate that elevated opioidergic function in 'reward-controlling' brain regions may be the driver of the elusive 'runner's high' (Boecker et al., 2008), it is possible that HVR rats are merely running to obtain a 'runner's high.' This idea is supported by my findings in Chapter 2, as well as previous findings that 1) antagonizing Oprm1 signaling locally in the NAc decreases running, suggesting the involvement of downstream Oprm1 activity in the acquisition of wheel running reward; and paradoxically 2) agonizing Oprm1 locally in the NAc decreases running, suggesting that rats may run to achieve an 'opioid high' that when artificially provided partially discourages the rat from running. Nonetheless, inherent differences in Oprm1 function may provide clues as to why certain humans repeatedly find pleasure in performing outlandish amounts of physical activity, while others seem to obtain no reward from physical activity. This hypothesis has been presented to explain running addiction in humans (Boecker et al., 2008).

The results from Chapter 2 also suggest that HVR and LVR lines may display differing preferences for drugs of abuse. It is well recognized that the neuromolecular pathways influencing wheel running also influence addiction and drug usage. For example, rats with a high avidity for wheel running are more motivated to self-administer cocaine than outbred rats with a low avidity for voluntary running (Larson and Carroll, 2005). However, the addition of a running wheel reduces cocaine self-administration compared to sedentary rats, highlighting the ability of physical activity to substitute for the reward derived from

cocaine (Zlebnik et al., 2012). With reference to Oprm1, naltrexone injection reduces ethanol consumption in a DA-dependent mechanism, as determined by measuring extracellular DA measurements *in vivo* in the NAc (Valenta et al., 2013). Likewise, drug-induced locomotor activity has long been known to be the result of elevated DA in the NAc following repeated drug injection, and drug-induced locomotor activity serves as a simple screening tool for testing the sensitivity of rodents to drugs of abuse (Wise and Bozarth, 1987) (Delfs et al., 1990). Similarly, my findings in Chapter 4 also support commonalties between molecular and structural changes that regulate drug addiction also mediate wheel-running behavior. Collectively, I believe a major outcome from this research could be to encourage future research into using exercise to prevent and/or treat drug addiction in patient populations. The concept of treating addicts with exercise has been extensively discussed, with the general census supporting future strategies to implement select exercise protocols to addicts recovering from various stages of addiction (Lynch et al., 2013). Ultimately, I speculate that the efficacy of exercise to treat drug addiction may relate to its ability to facilitate DA transmission as well as its ability to normalize dopaminergic and glutamatergic signaling and drug-induced epigenetic changes in chromatin structure in the cells of the reward system once addiction develops. The strong parallels between wheel running and drug addiction may make the HVR/LVR model an enticing model to study addiction and mental health.

While much of my discussion has focused on the inherent divergence in opioid and DA-related signaling between HVR and LVR rats, it is also important to consider the polygenic nature by which this model is built. For example, in Chapter 2 the lesion of DA neurons in the NAc of HVR rats with 6-OHDA only produced minor reductions in wheel running following injection. Further supporting, running distance had returned to level of

vehicle treated rats by two-weeks post-injection (Figure 2.5). While this finding again highlights the role of DA in contributing to the wheel running behavior in HVR rats, it also suggests DA is not essential to maintain high voluntary running levels. The ability of other neuromodulators to compensate for the loss of DA highlights the complex, polygenic control of voluntary physical activity. Nonetheless, these findings may have translational implication for uncovering the control of physical activity motivation as well as for developing strategies to help recovering drug addicts.

Future directions from this study include addressing opioidergic and dopaminergic contributions to wheel running with genetic manipulation models (e.g. overexpression of knockdown of Oprm1 or opioid ligands in the NAc or other mesolimbic brain nuclei in HVR or LVR rats) to study the direct contributions of opioid- and DA-related gene expression to high and low voluntary running phenotypes. For example, I hypothesize that knockdown of Oprm1 in the NAc will reduce wheel running in HVR rats, while overexpression of the Oprm1 ligand β-endorphin in the NAc will increase wheel running in LVR rats. Experiments in which opioid and DA receptors are simultaneously agonized and antagonized, respectively, in the NAc would further my conclusions about the necessity of the DA system for opioidergic influence on wheel running behavior. As mentioned previously, the neuromolecular mechanisms governing wheel running and addictive behaviors appear comparable. This suggests that HVR rats may be more prone to various drugs of abuse (e.g. ethanol, cocaine, etc). For example, our lab has identified parallelisms in cocaine-induced locomotor activity and anxiety-like behavior between HVR and LVR rats (Brown et al., 2015). Future experiments using HVR and LVR rats investigating the propensity for various drugs of abuse, and the ability of voluntary wheel running to compete with the reward

derived from drugs of abuse may further associate running and addictive neurocircuitry, and suggest exercise may be part of a viable treatment for recovering addicts. The assessment of dendritic density in the NAc and other brain regions in sedentary and wheel running HVR and LVR rats may also draw further parallels between running motivation and drug motivation.

Maternal Western diet and obesity may be key drivers of offspring physical activity

Further conjecture relates to an important clinical condition. Since the initial observations by Garrod during the turn of the last century that conferred the existence of inherited biochemical characteristics from family histories of alkaptonuria (Garrod, 1902), it has been increasingly appreciated that most common diseases involve not only discrete genetic and environmental causes, but also gene-environment interactions (Hunter, 2005). For example, Garrod proposed that the influences of diet and disease may mask some of the "inborn errors in metabolism" that were initially proposed as causes of disease. Indisputable evidence shows that genetic and environmental underpinnings cause obesity (Qi and Cho, 2008). Importantly, these genetic and environmental contributions to obesity are posited to cause transgenerational alterations in dopamine signaling and metabolic dysfunction (Heerwagen et al., 2010; Ong and Muhlhausler, 2011; Vucetic et al., 2012; Vucetic et al., 2010). Given that maternal obesity prevalence during pregnancy is as high as 38% (Yogev and Catalano, 2009), in Chapter 3 I sought to examine how environmental factors contributing to obesity during pregnancy potentially contribute to transgenerational genetic and behavioral changes in juvenile and young-adult offspring. Based upon previous findings that Oprm1 mRNA is increased and decreased in male and female juvenile and adult rats fed

a cafeteria-style diet, respectively (Ong and Muhlhausler, 2011) and upon my previous findings that Oprm1 mRNA positively associates with running distance (Ruegsegger et al., 2015), I hypothesized that male and female offspring of dams fed WD prior to and during pregnancy would have increased voluntary wheel running distance at juvenile ages but decreased wheel running distance by young adulthood. Additionally, I hypothesized changes in offspring wheel running would associate with increased and decreased Oprm1 mRNA expression during juvenile and adult ages, respectively. These hypotheses were the basis for the experiments performed in Chapter 3 of my dissertation.

Contrary to my hypothesis, maternal WD had no influence on offspring Oprm1 mRNA expression. Instead, my results from Chapter 3 suggest that gestational WD and maternal obesity lead to: 1) increased and decreased voluntary physical activity (i.e. wheel running) levels in juvenile and adult female offspring, respectively, while voluntary physical activity levels are unaltered in male offspring; 2) changes in the directionality of voluntary physical activity levels are complemented by changes in dopamine receptor mRNA and protein levels in the NAc and Lepr mRNA in the VTA; 3) differences in voluntary physical activity levels between SD and WD offspring appear independent of metabolic disease risk (e.g. increases in body fat and serum leptin and insulin); and 4) the lack of differences in F_2-offspring voluntary wheel running, adiposity, and dopamine receptor mRNA expression suggests the above differences are the result of direct in-utero exposure to maternal WD/obesity, rather than permanent transgenerational (potentially epigenetic) changes.

The link between enhanced DA action in the NAc and greater wheel-running behavior is well-established (Knab and Lightfoot, 2010). Therefore, I posit that this link may provide one basis by which gene-environment interactions influence voluntary physical

activity. Interestingly, a recent publication by Friend et al. (Friend et al., 2016) reported that chronic HFD exposure in mice reduces striatal Drd2 binding, which in turn leads to reduced wheel running. Thus, mechanistic links explaining alterations in wheel running behavior in response to direct or maternal overnutrition may stem from 'dysfunctional' dopaminergic signaling. Equally fascinating are similar findings that Drd2 knockdown in mice produces an obese phenotype compared to wild-type littermates that is primarily due to decreased energy expenditure rather and increased food intake (Beeler et al., 2016). Future studies in which dopamine receptor expression is restored in knockdown or knockout mice fed a WD or HFD may be fruitful in better providing a direct causal link between dopamine receptor function and abnormal wheel-running patterns following overnutrition.

Interestingly, I observed strong sex-specific differences in voluntary wheel running of offspring, with females from maternal WD fed dams having increased and decreased wheel running at juvenile and adult ages, respectively. Paradoxically, this observation is in line with human "quasi-experimental observations" such as the Dutch Hunger Study and Biafra famine (Hult et al., 2010; Stein et al., 2007; Yang et al., 2008) in which maternal and fetal undernutrition and growth restriction reprogram physical activity levels, specifically in females. Recent human epidemiologic data of Cameroonian children (Said-Mohamed et al., 2012) and Nordic adults (Andersen et al., 2009) also suggests that early-life nutritional status (both over- and undernutrition) can decrease physical activity in adulthood. Additionally, numerous pre-clinical rodent studies show that prenatal and postnatal nutritional status can lead to persistent changes in physical activity. In Wistar rats fed either *ad libitum* or at 30% of *ad libitum* intake, Vickers et al. (Vickers et al., 2003) observed consistently decreased locomotor behavior at 35, 145, and 420 days of age despite the presence of a healthy diet

during postnatal life. However, findings from Vickers et al. are severely limited in that activity was monitored for only 15 minutes in each animal and no data on body weight or body fat were reported. Bellinger et al. (Bellinger et al., 2006) studied the offspring of dams fed a low-protein diet during various stages of pregnancy and reported female-specific decreases in locomotor activity during adulthood. However, this effect was present in only one of four experimental groups studied. Additionally, the Bellinger et al. results are limited in that activity measurements were conducted for only 30 or 60 minutes and, surprisingly, locomotor activity was not markedly different between the light and dark cycle. Likewise, in female offspring of rat dams fed a lard-rich diet from before pregnancy through lactation displayed decreased physical activity at 180 days of age (Khan et al., 2003). Finally, significant contributions to understanding how maternal nutritional status influence physical activity levels have been made by Waterland and colleagues. Li et al. (Li et al., 2013) reported that postnatal overnutrition leads to decreased spontaneous physical activity in adult (180 days old) female offspring. Likewise, Baker et al. (Baker et al., 2015) unexpectedly observed that female, but not male, offspring of obese-agouti, viable-yellow dams displayed decreased spontaneous physical activity leading to adult-onset obesity.

Given the similarities of my data in Chapter 3 to previous human and rodent data demonstrating decreased physical activity in response to fetal undernutrition, I speculate that although the WD-fed dams in Chapter 3 were obese, F_1-offspring to WD dams were undernourished due to their diet providing low protein. This notion is supported by my observation in Chapter 3 that juvenile WD F_1 offspring had decreased body weight, as well as decreased bone mineral density (data not included), compared to SD offspring. Multiple publications have also reported similar reductions in body weight in juvenile offspring from

WD, or junk food, fed dams (Bayol et al., 2007; Ferezou-Viala et al., 2007; Ong and Muhlhausler, 2011; Rolls and Rowe, 1982).

A key observation in Chapter 3 was that increased and decreased wheel-running observed in F_1 WD female offspring associated with greater and lower levels of Drd1 in the NAc, respectively. This finding is matched by a publication in which maternal overnutrition, which like my results in Chapter 3 also resulted in reduced offspring body weight at juvenile ages, reported the up-regulation of DA-related mRNAs at 6 weeks of age, but down-regulation of DA-related mRNAs at 3 months of age (Ong and Muhlhausler, 2011). Future studies addressing the contributions of dopaminergic signaling to physical activity following maternal over- or under-nutrition will provide valuable insight into potential mechanisms by which maternal overnutrition interacts with genetics to dictate the motivation for physical activity.

Additionally, I noted novel observations among leptin, DA, and voluntary running distance. In Chapter 3, a significant negative association between Lepr mRNA expression in the VTA and Drd1 mRNA expression in the NAc in adult female offspring, and a significant negative association between serum leptin and wheel running distance in female offspring from WD dams. Likewise, Lepr mRNA in the VTA was significantly down- and up-regulated in 6- and 18-week-old F_1 female WD offspring, respectively. This observation is highly intriguing given that leptin receptor signaling is well characterized in DA neurons, primarily in the VTA, and previous reports highlighting leptin as a strong regulator or mesolimbic DA circuitry (Domingos et al., 2011; Fulton et al., 2006; Hommel et al., 2006). Fernandes et al. (Fernandes et al., 2015) demonstrated that intra-VTA leptin injection suppresses the rewarding effects of wheel running in mice via activation of STAT3 signaling

in mesolimbic DA neurons. Additionally, the same report found that the loss of STAT3 in DA neurons blunts DA overflow and function in the NAc, suggesting that leptin may influence the motivational and rewarding effects of wheel running. Equally fascinating are findings that maternal hyperleptinemia during pregnancy in mice increases spontaneous activity in adult female, but not male, offspring, (Pollock et al., 2015), and the offspring of diabetic and obese mothers display characteristic central leptin resistance (Chen et al., 2008; Steculorum and Bouret, 2011). Leptin levels inversely correlate with marathon run-time, after adjusting for BMI (Bobbert et al., 2012) and running performance (time and speed) in mice (Girard et al., 2007), also demonstrating that leptin could be a key regulator of the motivational and rewarding effects of running. My take on the above is future studies assessing the effect of intra-VTA leptin injection on wheel running in the offspring of SD and WD fed dams will further our understanding of how disruptions in mesolimbic leptin signaling influence physical activity levels.

In Chapter 3, I found minimal evidence for F_0 maternal WD/obesity leading to transgenerational transmission influencing adiposity, voluntary wheel running, or brain mRNA expression. Contrary to my results, previous reports have identified transgenerational increases in body size (Dunn and Bale, 2011), insulin resistance (Dunn and Bale, 2009; Huypens et al., 2016), and leptin resistance and obesity (Sun et al., 2012) following maternal HFD/obesity during pregnancy in rodents. Interestingly, other findings show that changes in F_1 and F_2 offspring in response to maternal HFD may be dependent on maternal obesity. For example, White et al. (White et al., 2009) tested the hypothesis that maternal HFD consumption, independent from obesity, predisposes offspring to obesity and increased metabolic risk factors in adulthood in Long-Evans rats. In their experiment, White et al. fed

female dams either a HFD, a low-fat diet (LFD), or a HFD with paired caloric intake to the LFD group (PF). Intriguingly, by 18 weeks of age, offspring of obese HFD dams displayed increases in body weight, body fat percentage, food intake, serum leptin, and insulin resistance compared to offspring from both LFD and PF dams, with no differences reported between offspring of non-obese LFD and PF dams (White et al., 2009). Collectively, these findings highlight the importance of maternal adiposity for offspring metabolic health; however, to my knowledge, similar assessments of brain mRNA and protein levels have not been made using similar models to dissect the influence of maternal HFD consumption versus maternal obesity on altered brain mRNA expression patterns.

Although I did not measure body fat percentage in F_1 breeders to create SD and WD F_2-offspring, no differences in body weight, body fat, or serum leptin were present between SD and WD F_1 female offspring, suggesting that F_1 SD and WD breeders did not differ in body fat percentage. Given the observations by White et al. (White et al., 2009), I speculate that the plausible lack of maternal obesity in F_1 breeders led to the lack of differences in physical activity and adiposity in F_2 SD and WD offspring. The observation that changes in physical activity and adiposity were not observed in the F_2 generation may have important public health ramifications. For example, my data suggest that healthy lifestyle choices (e.g. eating a healthy or 'standard' diet) may protect an F_2 offspring from predispositions to metabolic and behavioral 'dysfunction' that were inherited in F_1 offspring following F_0 obesity.

Unexpectedly, I did not observe differences in physical activity, dopamine or leptin receptor mRNA expression, or adiposity in F_2 WD, compared to SD, offspring. However, Dat mRNA was decreased in F_2 WD offspring, highlighting the ability for genetic

information to transmit across multiple generations. However, the lack of voluntary wheel running differences in F_2 offspring suggests Dat mRNA could have, at most, a minimal role in the regulation of voluntary physical activity levels compared to other NAc mRNAs whose expression patterns matched changes in voluntary running in both F_1 and F_2 offspring, such as Drd1 mRNA.

The preservation of dendritic density and dopaminergic activity in the nucleus accumbens could be paramount to preventing age-related declines in physical activity

Understanding the mechanisms causing physical activity to naturally decline throughout the life course will be valuable to the development of strategies or therapies to reduce human sedentary behavior. In Chapter 4, I performed transcriptomics (RNA-seq) analysis of the NAc of wheel running rats at 8 and 14 week of age, ages at which wheel running is at its max and have significantly declined by ~60% from max, respectively. Based upon previous findings that the loss of DA in the striatum associates with reduced physical activity levels in humans (Sallis, 2000), I hypothesized that dopamine-related mRNAs would be down-regulated in the NAc at 14 versus 8 weeks of age, however these results were severely limited by the lack of age-matched sedentary control rats, such that the influences of age and physical activity level could not be distinguished. Additionally, these RNA-seq efforts allowed me to observe other non-DA pathway transcriptomic differences within the NAc that existed between the 8 and 14 week-old comparisons in order to generate new hypotheses for future experiments using this model. My analysis of transcriptomic differences between 8 and 14 week-old running rats led to follow-up mRNA, protein, and histological experiments in both wheel running and sedentary rats at 8 and 14 weeks of age.

In addition, the Cdk5 inhibitor roscovitine was injected into the NAc to assess its role in modulating wheel-running behavior.

The above experimental approaches resulted in the following novel observations: 1) reductions in mRNAs critical for synaptic plasticity, and decreases in dendritic density in the NAc may be central to age-related changes in voluntary running, and 2) inhibition of Cdk5, a key regulator of dendritic spine density and DA transmission in the NAc, reduced wheel running.

In Chapter 4, I found that pathways and networks associated with cAMP/DARPP-32 signaling, PKA activity, and synaptic transmission were reduced from 14 vs. 8 weeks of age in wheel running rats. While the relative contributions of age and physical activity on the activity of these pathways could not be determined, follow-up analysis revealing age-dependent reductions in Cadm4 and Cdk5r2 (p39) mRNA and protein suggest that reductions in these pathways change with age. I found similar results for changes in MSN dendritic density, in which dendritic spine density was inherently reduced from 8 to 14 weeks of age, suggesting my notion that inherent reductions in NAc dendritic density could influence reductions in wheel running behavior. Interestingly, my observations were independent of changes in dopamine receptor expression, which I speculate may contribute to the differences in physical activity levels observed in Chapter 3. Equally interesting was the lack of mRNA differences related to glutamate, opioid, or serotonin activity between 8 and 14 weeks of age in my dataset. Together with the fact that MSNs (which comprise ~95% of the neurons in the ventral striatum) have strong dopaminergic presynaptic connections (Nishi et al., 2011; Yager et al., 2015), I speculate that changes in dopaminergic activity may 1) accompany reductions in voluntary physical activity, and 2) influence age-related changes in voluntary

physical activity. In addition, I noted that injection of the Cdk5 inhibitor roscovitine into the NAc reduced wheel running via a mechanism that appears to promote a 'substitution of reward' by replacing 'running reward' with 'drug reward' via potential alterations in dopamine signaling.

Importantly, these results suggest that the preservation of dendritic density and processes related to synaptic transmission and dopamine signaling in the NAc may help stave off age-related reductions in physical activity. Although paradoxical to some, one potential mechanism to increase MSN dendritic density, and thus possibly physical activity, appears to be physical activity itself (Figure 4.7). While exercise did not prevent age-related reductions in dendritic density, physical activity itself drastically increased dendritic spine density. Therefore, it is possible that continuous high levels of physical activity may induce alterations in dendritic density that in turn influence physical activity levels. However, my thoughts on this are purely speculative, and future experiments must be performed to test this hypothesis in experimental settings better suited to address this specific question than my design in Chapter 4. Interestingly, this hypothesis is supported by the literature. Werme et al. (Werme et al., 2002) posit that physical activity-induced increases in ΔFosB, potentially via its influence on Cdk5, promote further increased physical activity levels. In addition, strategies to maintain dopaminergic tone in the NAc may also help ward off age-related declines in physical activity. Reductions in dopaminergic function (e.g. decreased tissue DA contention, dopamine receptor expression, Dat expression, etc.) with advancing age (albeit most often studied in adulthood and the elderly) are associated with various behavioral abnormalities, including reductions in physical activity (Hemby et al., 2003; Ingram, 2000; Severson and Finch, 1980; Volkow et al., 1996; Volkow et al., 1998). Given the profound

influence of physical inactivity on the development on numerous mental health disorders, it is plausible that the mechanisms causing physical activity to decrease may also increase behavioral abnormalities (e.g. depression, anxiety, etc). Therefore, I speculate that strategies preserving dopaminergic function in the NAc could prevent age-related declines in physical activity as well as reduce mental health disorders that develop with advancing age.

Decreases in physical activity with aging have been observed in *C. elegans* (Herndon et al., 2002; Kirkwood and Finch, 2002), flies (Marden et al., 2003), dogs (Siwak et al., 2003), and humans (Black et al., 1996), suggesting it is a fundamental to many species. Equally as important is the age at which voluntary running and its accompanying molecular changes initially decrease. It is not surprising that physical activity levels are lessened in old age (Hallal et al., 2012), but it is rather surprising that numerous reports suggest that voluntary physical activity decreases during adolescent ages in humans (Troiano et al., 2008; Trost et al., 2002; Wolff-Hughes et al., 2014), as well as other species such as *C. elegans* and mice (Marck et al., 2016). Further, these findings suggest gene expression alterations during the period of adolescence and sexual maturity contribute to reductions in physical activity [rats reach sexual maturity at ~7 weeks of age, while humans reach sexual maturity at ~11-12 years of age (Sengupta, 2013)]. Sex hormones are key components in the regulation of various physiological parameters in both males and females, and thus have the potential to at least partially regulate physical activity. For example, estrogen (Garey et al., 2001; Park et al., 2016) and testosterone, likely through its aromatization to estrogen (McGinnis et al., 2007), are known to increase voluntary wheel running in rodents. Therefore, it is possible that gene expression changes occurring at the onset of sexual maturity have direct, or indirect, effects on the control of physical activity, however future experiments must be

performed to assess the influence of sexual maturation on wheel running behavior. For instance, estrogen can regulate several aspects of dopaminergic functioning, including DA release and pre- and post-synaptic receptor expression (Di Paolo, 1994). Given the relationship between dopaminergic function and sex hormone function, I postulate that future experiments that can alter the age at which sexual maturity occurs may alter the age at which voluntary physical activity initially declines. One potential strategy is the use of caloric restriction. As early as 1947, it was known that caloric restriction influenced the ages at which sexual maturity and senescence were reached (Ball et al., 1947). Caloric restriction also enhances evoked DA overflow in the NAc (Diao et al., 1997). Therefore, to test my notion that sexual maturity influences wheel-running behavior, future studies should assess the ability of early-life caloric restriction to delay both sexual maturity as well the age at which voluntary wheel running initially declines.

Additional future directions from this work include assessing using transgenic animals to access whether the overexpression of genes up-regulated at 8 weeks versus 14 weeks, and related to synaptic transmission and DA signaling, in the NAc either 1) increases wheel running, or 2) prevents age-related declines in wheel running. For example, I speculate that overexpression of either Cadm4 or p39, two molecules whose expression at the mRNA and protein levels inherently decreased from 8 to 14 weeks of age, whose expression correlate with wheel running distance, and whose functions influence DA action, in the NAc will both increase wheel running and prevent age-related declines in wheel running. Likewise, given the explicit usage of female rats in my study, it will be interesting to determine whether similar relationships are present in male rats.

Translational significance: linking dopamine signaling to physical activity regulation in humans

Strong cases have been made suggesting that wheel running in rodents is the best parallel to voluntary physical activity in humans (Eikelboom, 1999; Sherwin, 1998). Thus, I posit that the innate behavior mechanisms controlling voluntary wheel running in rodents provide the best approximate insight into mechanisms controlling physical activity in humans. Although the potential mechanisms of action differ between experiments (e.g. receptor expression in Chapters 2 and 3, synaptic function of MSN and Cdk5 activity in Chapter 4), the three aims of my dissertation suggest that alterations in NAc dopamine associate with, or contribute to, alterations in wheel running behavior.

It is well established that aerobic exercise increases DA release and metabolism in mesolimbic brain nuclei (Waters et al., 2005). In addition, while exercise itself increases DA action, these changes to the DA system can be accompanied by positive reinforcement in which DA, in turn, independently alters a behavior to seek additional rewarding responses (Wise, 2004). However, to my knowledge no studies have addressed the question "do differences in DA cause changes in physical activity in humans?" Such a question may be difficult to answer because of the next information. Locations of the brain networks controlling locomotion (e.g. striatum, nigrostriatal pathways) and reward (e.g. VTA, NAc) have multiple integrations via projections to multiple concurrent regions (e.g. ventral pallidum). Therefore, it is likely that the neuro-circuits controlling either the locomotor- or reward-associated aspects of physical activity do not act independently, and are/may be redundant and/or circular. This redundancy is partially highlighted by the common usage of

exercise as a treatment for depression, in which stimulating locomotor pathways appears to stimulate reward pathways (Dunn et al., 2005).

Non-pharmacological approaches may also influence physical activity. With new awareness of the health benefits of physical activity, wearable technologies quantifying physical activity level (e.g. Fitbit) have gained in popularity. One aspect of Fitbit and other accelerometers that I am perplexed by is "what is the biological mechanism by which a piece of plastic encourages someone to exercise?" Wearable technologies are becoming increasingly used in patients with neuropsychiatric illness to manage and/or reduce symptoms associated with numerous disease, and some "wearables" are thought to improve disease progression via dopaminergic modulation (Coffey and Coffey, 2016). Video games often produce variations in the dopaminergic system associated with reward dependence (Han et al., 2007). Thus, tools and devices that allow us to monitor our daily activities allow us to "gamify" our daily habits, and may provide ourselves with a "DA buzz" for reaching certain goals or milestones (e.g. step counts). To this end, I speculate one neuromolecular mechanism by which current strategies that improve physical activity adherence is via changes in dopaminergic signaling that associate physical activity with positive reward and physical inactivity with negative reward.

The epidemic levels of physical inactivity present health burdens that challenge those of any recognized chronic disease/disorder. Understanding the neuromolecular control dictating physical activity levels should help create strategies of therapies to reduce sedentary behavior. Regardless of the method of implementation, the findings from this dissertation suggest that inherent [e.g. selective breeding (Chapter 2) or age-related changes (Chapter 4)]

or environmentally produced [e.g. maternal WD (Chapter 3)] alterations in gene signatures which increase DA action in the NAc may be one vehicle to increase physical activity levels.

APPENDIX

APPENDIX A: Abstracts from 1st author original research manuscripts

Mu opioid receptor modulation in the nucleus accumbens lowers voluntary wheel running in rats bred for high running motivation

Ruegsegger GN, Toedebusch RG, Will MJ, Booth FW

Neuropharmacology. 2015; 97: 171-181.

The exact role of opioid receptor signaling in mediating voluntary wheel running is unclear. To provide additional understanding, female rats selectively bred for motivation of low (LVR) versus high voluntary running (HVR) behaviors were used. Aims of this study were 1) to identify intrinsic differences in nucleus accumbens (NAc) mRNA expression of opioid-related transcripts and 2) to determine if nightly wheel running is differently influenced by bilateral NAc injections of either the mu-opioid receptor agonist D-Ala2, NMe-Phe4, Glyo5-enkephalin (DAMGO) (0.25, 2.5 μg/side), or its antagonist, naltrexone (5, 10, 20 μg/side). In Experiment 1, intrinsic expression of Oprm1 and Pdyn mRNAs were higher in HVR compared to LVR. Thus, the data imply that line differences in opioidergic mRNA in the NAc could partially contribute to differences in wheel running behavior. In Experiment 2, a significant decrease in running distance was present in HVR rats treated with 2.5 μg DAMGO, or with 10 μg and 20 μg naltrexone between hours 0-1 of the dark cycle. Neither DAMGO nor naltrexone had a significant effect on running distance in LVR rats. Taken together, the data suggest that the high nightly voluntary running distance expressed by HVR rats is mediated by increased endogenous mu-opioid receptor signaling in the NAc,

that is disturbed by either agonism or antagonism. In summary, our findings on NAc opioidergic mRNA expression and mu-opioid receptor modulations suggest HVR rats, compared to LVR rats, express higher running levels mediated by an increase in motivation driven, in part, by elevated NAc opioidergic signaling.

Reduced metabolic disease risk profile by voluntary wheel running accompanying juvenile Western diet in rats bred for high and low voluntary exercise

Ruegsegger GN, Toedebusch RG, Braselton FJ, Roberts CK, Will MJ, Booth FW

Physiology & Behavior. 2015; 152: 47-55.

Metabolic disease risk is influenced by genetics and modifiable factors, such as physical activity and diet. Beginning at 6 weeks of age, rats selectively bred for high (HVR) versus low voluntary running distance (LVR) behaviors were housed in a complex design with or without voluntary running wheels being fed either a standard or Western (WD, 42% kcal from fat and added sucrose) diet for 8 weeks. Upon intervention completion, percent body fat, leptin, insulin, and mediobasal hypothalamic mRNAs related to appetite control were assessed. Wheel access led to differences in body weight, food intake, and serum leptin and insulin. Intriguingly, percent body fat, leptin, and insulin did not differ between HVR and LVR lines in response to the two levels of voluntary running, regardless of diet, after the 8 wk. experiment despite HVR eating more calories than LVR regardless of diet and voluntarily running 5-7 times further in wheels than LVR. In response to WD, we observed increases in Cart and Lepr mediobasal hypothalamic mRNA in HVR, but no differences in LVR. Npy mRNA was intrinsically greater in LVR than HVR, while wheel access led to greater Pomc and Cart mRNA in LVR versus HVR. These data suggest that despite greater consumption of WD, HVR animals respond similarly to WD as LVR as a result, in part, of their increased wheel running behavior. Furthermore, high physical activity in HVR may offset the deleterious effects of a WD on adiposity despite greater energy intake in this group.

Rapid alterations in perirenal adipose tissue transcriptomic networks with cessation of voluntary running

Ruegsegger GN*, Company JM*, Toedebusch RG, Roberts CK, Roberts MD, Booth FW

PLoS One. 2015; 10: e0145229.

*authors contributed equally to manuscript

In maturing rats, the growth of abdominal fat is attenuated by voluntary wheel running. After the cessation of running by wheel locking, a rapid increase in adipose tissue growth to a size that is similar to rats that have never run (i.e. catch-up growth) has been previously reported by our lab. In contrast, diet-induced increases in adiposity have a slower onset with relatively delayed transcriptomic responses. The purpose of the present study was to identify molecular pathways associated with the rapid increase in adipose tissue after ending 6 wks of voluntary running at the time of puberty. Age-matched, male Wistar rats were given access to running wheels from 4 to 10 weeks of age. From the 10th to 11th week of age, one group of rats had continued wheel access, while the other group had one week of wheel locking. Perirenal adipose tissue was extracted, RNA sequencing was performed, and bioinformatics analyses were executed using Ingenuity Pathway Analysis (IPA). IPA was chosen to assist in the understanding of complex 'omics data by integrating data into networks and pathways. Wheel locked rats gained significantly more fat mass and significantly increased body fat percentage between weeks 10-11 despite having decreased food intake, as compared to rats with continued wheel access. IPA identified 646 known transcripts differentially expressed ($p < 0.05$) between continued wheel access and wheel locking. In wheel locked rats, IPA revealed enrichment of transcripts for the following functions: extracellular matrix, macrophage infiltration, immunity, and pro-inflammatory.

These findings suggest that increases in visceral adipose tissue that accompanies the cessation of pubertal physical activity are associated with the alteration of multiple pathways, some of which may potentiate the development of pubertal obesity and obesity-associated systemic low-grade inflammation that occurs later in life.

Hypothalamic Npy mRNA is correlated with increased wheel running and decreased body fat in calorie-restricted rats

Ruegsegger GN, Speichinger KR, Manier JB, Younger KM, Childs TE, Booth FW

Neuroscience Letters. 2016; 618: 83-88.

The neuro-molecular mechanisms that regulate the relationship between physical activity level, energy homeostasis regulation, and body fat are unclear. Thus, we aimed to investigate the relationship between mRNAs in the hypothalamic arcuate nucleus(ARC) related to energy homeostasis, wheel running distance, and body fat in *ad lib* (AL) and calorie-restricted (CR) growing rats. We hypothesized that changes in select mRNAs (Pomc, Cart, Agrp, Npy, Lepr, Insr, Mc4r, Ampk, Sirt1, Sirt3) in CR would be associated with decreases in body fat percentage and increased wheel running behavior. Male Wistar rats were given access to voluntary running wheels at 4 weeks of age and randomized into AL (n = 8) and CR (70% of AL; n = 7) groups at 5 weeks of age until study termination at 12 weeks of age. Body composition, serum leptin, insulin, and adiponectin, and ARC mRNA expression in AL and CR rats were assessed and correlated with week-12 running distance to examine potential relationships that may exist. By 12 weeks of age, wheel running was increased ∼3.3-fold ($p = 0.03$) while body fat percentage was ∼2-fold lower in CR compared to AL ($p = 0.001$). Compared to AL, ARC Npy mRNA expression was ∼2-fold greater in CR ($p = 0.02$), while Lepr, Insr, Ampk, and Sirt1 mRNA were additionally increased in CR ($p < 0.05$). Significant correlations existed between ARC Npy mRNA levels versus week-12 wheel running distance ($r = 0.81$, $p = 0.03$), body fat ($r = −0.93$, $p < 0.01$), and between body fat and wheel running ($r = −0.83$, $p = 0.02$) in CR, but not in AL. These results reveal possible mechanisms by which fat-brain crosstalk may influence physical activity during

energy deficit. These data suggest that below a ‘threshold’ fat content, body fat may drive activity levels, potentially through hypothalamic Npy action.

Left ventricle transcriptomic analysis reveals connective tissue accumulation associates with initial age-dependent decline in $\dot{V}O_{2peak}$ from its lifetime apex

Ruegsegger GN, Toedebusch RG, Braselton JF, Childs TE, Booth FW

Physiological Genomics. 2017; 49: 53-66.

Peak oxygen consumption ($\dot{V}O_{2peak}$) strongly predicts morbidity and mortality better than other established risk factors, yet mechanisms associated with its age-associated decline are unknown. Our lab has shown that $\dot{V}O_{2peak}$ first begins to decrease at the same age of 19-20 weeks old in both sedentary and wheel-running, female Wistar rats (Toedebusch et al., *Physiol Genomics*. 48:101-15, 2016). Here, we employed a total systemic approach using unsupervised interrogation of mRNA with RNA-sequencing. The purpose of our study was to analyze transcriptomic profiles from both sedentary (SED) and wheel-running (RUN) conditions as a strategy to identify pathways in the left ventricle that may contribute to the initial reductions in $\dot{V}O_{2peak}$ occurring between 19 and 27 weeks of age. Transcriptomic comparisons were made within both SED and RUN rats between 19 and 27 wks (n= 5-8). Analysis of mRNAs shared in SED and RUN between 19 and 27 wks found 17 up-regulated (e.g. Adra1d, Rpl17, Xpo7) and 8 down-regulated (e.g. Cdo1, Ctfg, Sfrp1) mRNAs, at 19 wks, respectively. Furthermore, bioinformatics analysis of mRNAs common to SED and RUN produced networks suggestive of increased connective tissue development at 27 vs. 19 wks. Additionally, Ctfg mRNA was negatively associated with $\dot{V}O_{2peak}$ in both SED and RUN ($p< 0.05$). In summary, transcriptomic analysis revealed mRNAs and networks associated with increased connective tissue development, decreased α-adrenergic activity, and decreased protein translation in the left ventricle that could, in part, potentially influence the initiation of the lifelong reduction in $\dot{V}O_{2peak}$, independent of physical activity levels.

APPENDIX B: Abstracts from co-authored original research manuscripts

Comparative adaptations in oxidative and glycolytic muscle fibers in a low voluntary wheel running rat model performing three levels of physical activity

Hyatt HW, Toedebusch RG, **Ruegsegger GN**, Mobley CB, Fox CD, McGinnis GR, Quindry JC, Booth FW, Roberts MD, Kavazis AM

Physiological Reports. 2015; 3: pii: e12619.

A unique polygenic model of rat physical activity has been recently developed where rats were selected for the trait of low voluntary wheel running. We utilized this model to identify differences in soleus and plantaris muscles of sedentary low voluntary wheel running rats and physically active low voluntary wheel running rats exposed to moderate amounts of treadmill training. Three groups of 28-day-old male Wistar rats were used: (1) rats without a running wheel (SEDENTARY, n = 7), (2) rats housed with a running wheel (WHEEL, n = 7), and (3) rats housed with a running wheel and exercised on the treadmill (5 days/week for 20 min/day at 15.0 m/min) (WHEEL + TREADMILL, n = 7). Animals were euthanized 5 weeks after the start of the experiment and the soleus and plantaris muscles were excised and used for analyses. Increases in skeletal muscle gene expression of peroxisome proliferator-activated receptor gamma coactivator 1 alpha and fibronectin type III domain-containing protein 5 in WHEEL + TREADMILL group were observed. Also, WHEEL + TREADMILL had higher protein levels of superoxide dismutase 2 and decreased levels of oxidative damage. Our data demonstrate that the addition of treadmill training induces beneficial muscular adaptations compared to animals with wheel access alone. Furthermore, our data expand our understanding of differential muscular adaptations in response to exercise in mitochondrial, antioxidant, and metabolic markers.

AMPK agonist AICAR delays the initial decline in lifetime-apex $\dot{V}O_{2peak}$, while voluntary wheel running fails to delay its initial decline in female rats

Toedebusch RG, **Ruegsegger GN**, Braselton JF, Heese AJ, Hofheins JC, Childs TE, Thyfault JP, Booth FW

Physiological Genomics. 2016; 48: 101-115.

There has never been an outcome measure for human health more important than peak oxygen consumption (V̇o2 peak), yet little is known regarding the molecular triggers for its lifetime decline with aging. We examined the ability of physical activity or 5 wk of 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) administration to delay the initial aging-induced decline in lifetime-apex V̇o2 peak and potential underlying molecular mechanisms. Experiment 1 consisted of female rats with (RUN) and without (NO RUN) running wheels, while experiment 2 consisted of female nonrunning rats getting the AMPK agonist AICAR (0.5 mg/g/day) subcutaneously for 5 wk beginning at 17 wk of age. All rats underwent frequent, weekly or biweekly V̇o2 peak tests beginning at 10 wk of age. In experiment 1, lifetime-apex V̇o2 peak occurred at 19 wk of age in both RUN and NO RUN and decreased thereafter. V̇o2 peak measured across experiment 1 was ~25% higher in RUN than in NO RUN. In experiment 2, AICAR delayed the chronological age observed in experiment 1 by 1 wk, from 19 wk to 20 wk of age. RUN and NO RUN showed different skeletal muscle transcriptomic profiles both pre- and postapex. Additionally, growth and development pathways are differentially regulated between RUN and NO RUN. Angiomotin mRNA was downregulated postapex in RUN and NO RUN. Furthermore, strong significant correlations to V̇o2 peak and trends for decreased protein concentration supports angiomotin's potential importance in our model. Contrary to our primary hypothesis, wheel

running was not sufficient to delay the chronological age of lifetime-apex V̇o2 peak decline, whereas AICAR delayed it 1 wk.

Effects of intrinsic aerobic capacity and ovariectomy on voluntary wheel running and nucleus accumbens dopamine receptor gene expression

Park YM, Kanaley JA, Padilla J, Zidon T, Welly RJ, Will MJ, Britton SL, Koch LG, **Ruegsegger GN**, Booth FW, Thyfault JP, Vieira-Potter VJ.

Physiology & Behavior. 2016; 164: 383-389.

Rats selectively bred for high (HCR) and low (LCR) aerobic capacity show a stark divergence in wheel running behavior, which may be associated with the dopamine (DA) system in the brain. HCR possess greater motivation for voluntary running along with greater brain DA activity compared to LCR. We recently demonstrated that HCR are not immune to ovariectomy (OVX)-associated reductions in spontaneous cage (i.e. locomotor) activity. Whether HCR and LCR rats differ in their OVX-mediated voluntary wheel running response is unknown.

PURPOSE: To determine whether HCR are protected from OVX-associated reduction in voluntary wheel running.

METHODS: Forty female HCR and LCR rats (age ~27weeks) had either SHM or OVX operations, and given access to a running wheel for 11weeks. Weekly wheel running distance was monitored throughout the intervention. Nucleus accumbens (NAc) was assessed for mRNA expression of DA receptors at sacrifice.

RESULTS: Compared to LCR, HCR ran greater distance and had greater ratio of excitatory/inhibitory DA mRNA expression (both line main effects, $P<0.05$). Wheel running distance was significantly, positively correlated with the ratio of excitatory/inhibitory DA mRNA expression across animals. In both lines, OVX reduced wheel running ($P<0.05$). Unexpectedly, although HCR started with significantly greater voluntary wheel running, they had greater OVX-induced reduction in wheel running than LCR such that no differences

were found 11 weeks after OVX between HCROVX and LCROVX (interaction, $P<0.05$). This significant reduction in wheel running in HCR was associated with an OVX-mediated reduction in the ratio of excitatory/inhibitory DA mRNA expression.

CONCLUSION: The DA system in the NAc region may play a significant role in motivation to run in female rats. Compared to LCR, HCR rats run significantly more, which associates with greater ratio of excitatory/inhibitory DA mRNA expression. However, despite greater inherent motivation to run and an associated brain DA mRNA expression profile, HCR rats are not protected against OVX-induced reduction in wheel running or OVX-mediated reduction in the ratio of excitatory/inhibitory DA receptor mRNA expression. OVX-mediated reduction in motivated physical activity may be partially explained by a reduced ratio of excitatory/inhibitory DA receptor mRNA expression for which intrinsic fitness does not confer protection.

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VITA

Gregory Neal Ruegsegger was born April 5, 1991 in Billings, Montana to Bill and Linda Ruegsegger. From an early age, Greg loved the outdoors and the freedom of the Montana wilderness, and he could regularly be found playing ice hockey, golf, or at one of the areas many lakes. He graduated from Skyview High School in Billings, MT in 2009, and received a B.S. in Exercise Science from Montana State University in 2013. As an undergraduate, Greg worked with Dr. Mary Miles to investigate the inflammatory responses accompanying various exercise and dietary interventions. Greg received his Ph.D. from the Department of Biomedical Sciences at the University of Missouri in 2017. As a graduate student, Greg worked with Dr. Frank Booth to investigate mechanisms influencing the motivation for physical activity, as well as conduct studies assessing adipose tissue responses to physical inactivity. Greg looks forward to establishing a laboratory in which he can discovery the health-promoting mechanisms of physical activity as well as motivate and inspire future scientists.